NUCLEOTIDE SEQUENCES SPECIFIC FOR MOUSE MAMMARY TUMOR VIRUS IN THE DNA OF MOUSE TISSUES

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NUCLEOTIDE SEQUENCES SPECIFIC FOR MOUSE MAMMARY TUMOR VIRUS IN THE DNA OF MOUSE TISSUES.

Edward Robert Medeiros

Under the supervision of Dr. H. E. Varmus and Dr. J. M. Bishop

Inbred strains of mice with a high incidence of mammary tumors have been found to harbor a mammary tumor virus(es) which is normally transmitted through the milk of lactating females. In addition, these mouse strains may contain mammary tumor virus-like information which is transferred via the gametes, since the removal of the milk agent can reduce but does not eliminate the occurrence of mammary tumors. Mice bred for their low incidence of mammary tumors do not normally transmit a milk born virus, yet recent experiments have indicated the possible genetic transmission of MMTV genes in these mice also. To investigate the inheritance of MMTV genes and their possible relationship to the incidence of mammary tumors, the DNA from "high" (RIII, C3H, GR) and "low" (BALB/c, C3HeB/Fe, C57BL/6) mammary tumor incidence strains of mice was examined for the presence of mouse mammary tumor virus-specific sequences, the number of viral copies/cell and the extent of viral genome representation by hybridization with MMTV specific DNA or RNA. Double and single stranded MMTV DNA was synthesized by virion associated RNA directed DNA and DNA directed DNA polymerase. Mouse DNA was assessed for virus sequences by its ability to accelerate the reassociation of MMTV double stranded DNA and by

its ability to accelerate the reassociation of MMTV double stranded DNA and by its capacity to hybridize the MMTV single stranded complementary DNA (cDNA) or MMTV RNA. The number of viral copies in the DNA from each mouse strain was estimated by the rate of accelerated reassociation of double stranded DNA or comparing the rate of hybridization of MMTV cDNA with the rate of reassociation for mouse "unique sequence" DNA. The degree of MMTV genome representation in cell DNA was appraised by the extent of hybridization achieved with MMTV RNA under conditions of vast DNA excess. The fidelity of hybrids formed between MMTV cDNA or MMTV RNA and cellular DNA was examined to estimate possible genotypic differences between MMTV genes carried by each of the mouse strains.

The principal findings of this investigation were:

(1) all of the mouse strains studied contain MMTV specific information in their DNA;

(2) virus specific information seems to be integrated into the mouse cell DNA by covalent bonds;

(3) BALB/c mice appear to have fewer viral sequences in their DNA than other mouse strains;

(4) no significant differences in viral copy numbers could be found between certain "high" (RIII, C3H) and "low" (C3HeB/Fe, C57BL/6) incidence mouse strains;

(5) GR mice appear to have two to three times more viral sequences in their genomes than other mouse strains;

(6) viral genes present in DNA from various mouse strains have some heterogeneity in nucleotide sequences;

(7) genotypic differences exist between the mammary tumor virus released from GR (MMTV-P) and BALB/c (MMTV-O) tumor cells;

(8) GR cell DNA appears to contain a majority, if not all, viral sequences for MMTV-P and MMTV-O viruses.

The results indicate that although all mice appear to contain MMTV specific sequences in their genomes, the mere presence of these sequences or their frequency of occurrence does not correspond to the incidence of mammary tumor development in a particular strain of mice.

Approved Harold Elliott Varmus
J M Bishop

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INTRODUCTION

Since early in the twentieth century evidence has been accumulating to suggest that all mice carry oncogenic information responsible for the development of mammary tumors, and that this information can be transmitted genetically from generation to generation.

The inheritable nature of mammary cancers in mice was first established in 1911. By mating the offspring of cancerous or non-cancerous animals, Murray (1911) was able to demonstrate that female mice with an ancestry of mammary tumors were more likely to develop mammary neoplasms than those with no familial history of cancer. The importance of genotype in the genesis of mouse mammary cancer was further established through genetic inbreeding. In America, Little and Strong developed several strains of mice with a "high" (C3H, A, DBA) or a "low" (CBA, BALB/c, C57BL, I) mammary tumor incidence by selecting for and against mammary tumors (Heston, 1945). Similar "high" mammary tumor strains were also developed in France (RIII, Dobrovolskaia-Zanadskaia, 1933), Holland (GR, Muhlbock, 1965) and Japan (DD, Heston et al., 1964).

By 1933 the staff of Roscoe B. Jackson Memorial Laboratory, headed by Dr. D. D. Little, had begun a series of experiments to examine the genetic inheritance of mammary tumors among mice of inbred strains (Staff, Jackson Memorial Laboratory, 1933). Reciprocal crosses between "high" and "low" mammary tumor mouse strains consistantly showed that F_1 females with mothers from "high" tumor strains developed

mammary carcinomas at a similar high incidence. However, females whose fathers were from "high" tumor strains did not demonstrate a high incidence of mammary tumors. Furthermore, a maternal influence on the development of mammary carcinomas could be carried through F_2 and back-cross generations (Murray and Little, 1935a,b). Since females from "high" and "low" mammary tumor incidence strains contained the same genotype, it was apparent that this maternal effect on mammary tumor development was not transmitted through the chromosomes but was due to an extra-chromosomal factor capable of being passed by the female to her offspring.

An extra-chromosomal maternal influence on the development of mouse mammary tumors was independently demonstrated by Korteweg in Holland, who proposed three possible mechanisms of transmission: cytoplasmic, intrauterine and milk. He favored the first mechanism, citing the distribution of plastids in the cytoplasm of plant cells (Korteweg, 1936). But Bittner (1936,1940) soon demonstrated the milk transmisison of a "mammary tumor agent". He showed that foster nursing A-strain mice (high tumor strain) on lactating females from strains CBA or C57BL (low tumor strains) reduced the percentage of mammary tumors from 96% to 8%. Also, F_1 generations from foster nursed mice remained relatively free of mammary tumors if nursed by their natural mothers; but when nursed by their A-strain grandmothers, the incidence of mammary tumors returned to 88%. Further experimentation by Bittner and his colleagues led him to make the following statement: "Since the milk agent, normally obtained by nursing, is filterable and sedimentable, can propagate in

the living cell and is an antigenic entity, it seems logical to classify it with filterable viruses, " (Bittner, 1946/47).

Today Bittner's "milk agent" (synonomous with mammary tumor inciter, MTI, milk-factor, Bittner virus) is known as mouse mammary tumor virus (MMTV) and is classified with the RNA tumor viruses. When viewed by the electron microscope, mouse mammary tumor virus has a distinctive morphology (B type, Bernhard, 1958). Virus particles are generally round (100-130 nm in diameter) with a surface membrane containing numerous spikes or projections (87-103A long) with circular knobs (45-65A in diameter) on their ends. They have an eccentrically located electron-dense nucleoid (65-85nm in diameter) composed of a helical ribonucleoprotein core surrounded by a lipopolysaccharide nucleoid capsule (Sarkar et al., 1971a,b; Sarkar and Moore, 1974). Viral particles contain 27-31% lipid and approximately 1% nucleic acid by dry weight. The nucleic acid is composed primarily of single stranded RNA with a GC content of 52% and a molecular weight of $10\text{-}12 \times 10^6$ daltons (Lyons and Moore, 1965; Duesberg and Blair, 1966). Treatment with heat or DMSO reduces the size of the RNA from 70S to 36-37S, as determined by velocity sedimentation analysis (Duesberg and Cardiff, 1968). This molecular conversion is characteristic of all RNA tumor viruses and indicates possible subunit structure of the viral genome. MMTV also contains an RNA directed DNA polymerase activity (reverse transcriptase) which requires a magnesium cation for optimum polymerization. B particles are not found intracellularly but can be seen budding from micro-villi in mouse mammary carcinomas and in normal glands of high tumor strain

mice (Moore, 1962; Feldman, 1963). They have been found in the epididimus (Smith, 1967), thymus (Hollman and Varley, 1967), in lung and brain tumors (Moore et al., 1969; Calafat, 1969), and in all infectious milk preparations (Moore, 1963). Intracytoplasmic A particles (donut shaped, 71nm in diameter, electron-lucent center, Smith and Wivel, 1973) have been seen in mammary tissues producing B particles. Because of their similar appearance to nucleoids of immature B particles, and because they contain MMTV specific antigens, they have been suggested as precursors in the formation of B particles (Smith and Wivel, 1973). Their role in MMTV development is unclear, however, since they may or may not appear in tissues producing B particles. In addition, large numbers of A particles are often seen in the cytoplasm of Leydig tumor cells, which produce no extra-cellular virus, and in leukemias and lymphomas, which actively produce C type particles (Nowinski et al., 1971; Tanaka et al., 1972).

Following the discovery of Bittner's agent, its actual role in mammary neoplasia remained somewhat controversial. "Virus free" mouse lines (C3Hf, RIIIf, C3HeB) obtained by foster nursing or by transplantation of ova from "high" to "low" incidence strains of mice continued to develop a significant number of spontaneous mammary tumors (30-40%) in old age (Heston et al., 1950; Dmochowski and Greg, 1957). Unsuccessful attempts to demonstrate virus in these tumors through bioassay led to the conclusion that virus was responsible for an accelerating effect but was not essential for mammary tumor development (Muhlbock, 1956). When the nodules or tumors from "virus free" animals

were examined by electron microscopy, however, they contained abundant A and B type particles (Pitelka et al., 1964; DeOme et al., 1966; Calafat and Hageman, 1968). Although these mice were not free of virus, attempts to transfer infection with milk or tumor extracts were unsuccessful (Muhlbock, 1956). After reviewing the evidence for and against viral involvement in the induction of "spontaneous" mouse mammary tumors, Moore (1963) proposed that mouse mammary tumor virus could go "underground" by in some way becoming integrated with the host and could re-emerge later when factors permitted, so that tumor formation could occur. Similar theories suggesting a lysogenic state or "virogeny" for oncogenic viruses had been proposed by Lwoff (1960). This concept was carried one step further by Pitelka et al. (1964) who showed by reciprocal crosses between BALB/c and C3Hf mice that F_1 females from either cross had approximately the same mammary tumor incidence as their C3Hf parent. They concluded that C3H mice had two viruses: one which could be transmitted by milk and one which could be transmitted genetically. Pitelka et al. (1965) finally succeeded in isolating the mammary tumor virus carried by C3Hf mice through a series of transplantation experiments. Mammary tissue from BALB/c mice was placed into the gland free fat pads of BALB/c X C3Hf females and allowed to become infected. The infected glands were then transferred back to BALB/c mice where virus was transmitted to the newborn through the milk. Mice infected with this virus developed a large number of hyperplastic alveolar nodules (HAN) at an early age which rarely developed into tumors. This was in contrast to the milk-borne virus of C3H mice which caused the

development of a large number of HAN at an early age, a large proportion of which progressed into tumors. DeOme et al. (1966) called this virus "nodule inducing virus" (NIV) to distinquish it from Bittner's agent and reaffirmed its genetic transmission in C3Hf and C3H mice (Nandi, 1966).

The genetic transmission of another mammary tumor virus was soon demonstrated by Muhlbock and Bentvelzen (1968) with a European inbred strain of mice, GR. Muhlbock's GR mice were infected with an MMTV which caused pregnancy responsive tumors called "plaques" (Foulds, 1956) in 100% of the female mice[1]. These tumors developed during pregnancy and regressed following parturition only to reappear again during the following pregnancy. Plaques often developed into neoplasms which no longer showed this pregnancy dependence. Induction of plaques appeared to be characteristic of the virus, since this type of lesion was formed when BALB/c or C57BL mice were infected by foster nursing or injection with purified virus. When C3H mice were foster nursed by C57BL females they no longer transmitted the mammary tumor agent in their milk, and there was a reduction in the incidence of mammary tumors among the female progeny. In contrast, when GR mice were foster nursed by C57BL or when their fertilized ova were transplanted into the uteri of C57BL mice, their offspring still maintained a 90% incidence of mammary tumors with the release of active virus in their milk (Muhlbock, 1965). A series of genetic experiments by Muhlbock and Bentvelzen (1968)

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[1] Hyperplastic alveolar nodules are not pregnancy dependent.

led them to conclude that GR mice harbored a virus that could be transmitted horizontally through the milk of lactating females or could also be transmitted vertically through the gametes.

By 1970 all strains of mice which had a high incidence of mammary tumor development (RIII, C3H, GR, DBA, A) were shown to contain a mammary tumor virus which was normally transmitted through the milk of lactating mice. In addition, some were shown to harbor a mammary tumor virus which was passed via the gametes and which may (GR) or may not (C3Hf, RIIIf) be transmitted through the milk (Bentvelzen et al., 1970). Evidence also indicated a genetically transmitted virus in "low" mammary tumor strains such as BALB/c, C57BL and 020.

BALB/c mice rarely develop mammary tumors before one year of age, but mammary carcinomas do appear in 20-30% of old BALB/c breeding females (Deringer, 1965; Heston and Vlahakis, 1971; Hilgers et al., 1973; Hageman et al., 1972; Moore et al., 1974). Early attempts at finding B particles in these tumors were unsuccessful. However, Bentvelzen in Holland and Pillsbury and Moore in America (Bentvelzen et al., 1970) recently reported such particles in BALB/c tumors. The virus has not been transmitted by milk, but injection of extracts from these tumors into new born BALB/c mice causes an early appearance of a large number of mammary neoplasms (Bentvelzen, 1972).

C57BL mice generally show less than a 1% incidence of mammary tumors even in old breeding mice (Gaff et al., 1949; Heston and Valahakis, 1971; Dux, 1972). Mammary tumors can be induced in these mice by treatment with X rays followed by extensive stimulation with

hormones (Boot et al., 1971). No B particles have been seen in these tumors, but such particles have been seen in a chemically induced C57BL brain tumor (Bentvelzen, 1970). C57BL mice treated with X rays followed by the administration of urethane in their drinking water develop a large number of lymphomas. MMTV antigens could be found in the bone marrow, spleens and lymphoid tissue of these X ray-urethane treated animals (Bentvelzen, 1972). Similar X ray and urethane treatment induced a mammary tumor virus in 020 mice which normally have no spontaneous mammary tumors (Timmermans et al., 1969).

Evidence for the presence of virus in "low" as well as "high" mammary tumor strains of mice has led Bentvelzen to conclude that all mice carry a mammary tumor virus. Bentvelzen et al. (1970) have proposed that MMTV is composed of several strains which can be classified according to their virulence, antigenic properties and the type of mammary tumor they induce (see Table I). To explain differences in the extent of viral gene expression in the various strains of mice, Bentvelzen (1972) advanced the idea of a "germinal provirus" present in all mice and transmitted vertically through their gametes. Expression of the "germinal provirus" would be subject to the normal cell regulatory mechanisms of the host strain, and the induction of virus by hormones, old age, X ray or chemical carcinogens would occur by interference with the normal control of these proviral genes. He further suggested that infection with milk borne virus would result in the synthesis of a "somatic provirus" similar to the DNA provirus proposed by Temin (1963). It would be synthesized through a viral RNA

directed DNA polymerase and could possibly become integrated into the host cell genome. The expression of integrated "somatic provirus" would be influenced by the same host genes governing the expression of "germinal provirus". And the extent of virus synthesis would depend upon the site of integration and genotype of the infected mouse.

A similar theory has also been proposed by Huebner and Todaro (1969; Todaro and Huebner, 1972) to explain evidence for C type viral information in normal cells. They claim that all cells contain genes which code for oncogenic information (oncogenes) and genes which code for viral information (virogenes). Like "germinal provirus", these oncogenes and virogenes are regulated by normal cell control mechanisms which are subject to alteration by old age, X ray and chemical carcinogens. Since C type RNA tumor viruses contain oncogenic information, they suggest that the oncogene is a part of the virogene or is acquired by the virogene at a high frequency. Evidence to support this hypothesis comes from the induction of viral antigens in normal cells (Huebner and Todaro, 1969); from induction of leukemia viruses from apparently uninfected chicken or mouse cells (Weiss et al., 1971; Lowey et al., 1971), and from molecular hybridization of viral nucleic acids with normal cell DNA (Varmus et al., 1971, 1972a,b; Gelb et al., 1971a, 1973).

Temin (1970, 1971a, 1972) has advanced another theory, "the protovirus hypothesis", which encompasses evidence for viral information in normal cells as well as evidence for the addition of new viral information upon infection and transformation with sarcoma viruses (Baluda

and Nayak, 1970; Rosenthal et al., 1971; Hansen, 1972; Neiman, 1972). He postulates that a pre-viral or protoviral sequence of genes is present in all normal cells, which can evolve into a leukosis or sarcoma virus within a few cell divisions. This evolution requires the transcription of protovirus sequences into RNA which is transcribed into DNA via an RNA directed DNA polymerase and becomes integrated into the cell genome. This process can be repeated several times with the addition of new protoviral or cellular sequences until an RNA tumor virus is produced. The protovirus hypothesis differs from the oncogene hypothesis in that it provides a method for the formation of new DNA sequences besides allowing for the activation and de-activation of pre-existing genes. Furthermore, animals within the same species may vary in the amount and extent of viral sequences present in their genomes.

The germinal-somatic provirus, oncogene and protovirus theories for genetic inheritance of viral information raise the following questions in regard to mouse mammary tumor virus inheritance: Do all mice contain MMTV information? If so, is MMTV-specific information covalently linked to the cell genome? How many copies of viral sequences are present in different strains of mice? What percentage of the viral genome is represented in the DNA from various mouse strains? Are there discernable differences between MMTV genes carried by each of the mouse strains? Are there additional MMTV sequences in cellular DNA following infection? Are additional sequences organ specific? What regulates the expression of viral genes?

The research presented in this dissertation represents an attempt to answer some of these questions through the use of nucleic

acid hybridization techniques. DNA from "high" (RIII, C3H, GR) and "low" (BALB/c, C3HeB/Fe, C57BL/6) mammary tumor incidence strains of mice was examined for the presence of mouse mammary tumor virus-specific sequences, the number of viral copies/cell and the extent of viral gene representation in cell DNA by hybridization with MMTV specific DNA or RNA. Double or single stranded MMTV DNA was synthesized by virion associated RNA directed DNA and DNA directed DNA polymerase in the absence (double stranded DNA) or presence (single stranded DNA) of actinomycin D (Temin and Mizutani, 1970; Baltimore, 1970; Spiegelman et al., 1970; Garapin et al., 1973). Relatively small pieces (4-10S; Bishop et al., 1973) of double or single stranded DNA are synthesized by these polymerases and can be separated by fractionation on hydroxylapatite (Fanshier et al., 1971). Mouse DNA was assessed for virus sequences by its ability to accelerate the reassociation of MMTV double stranded DNA (Gelb et al., 1971b), and by its capacity to hybridize with MMTV single stranded complementary DNA (cDNA) or MMTV RNA. The number of viral copies in the DNA from each mouse strain was estimated by the rate of accelerated reassociation of DS-DNA or by comparing the rate of hybridization of MMTV cDNA with the rate of reassociation for mouse "unique sequence" DNA. The extent of MMTV genome representation in cell DNA was appraised by the extent of hybridization achieved with MMTV RNA under conditions of vast DNA excess (Gelderman et al., 1968; Melli et al., 1971). The fidelity of hybrids formed between MMTV cDNA or MMTV RNA and cellular DNA was examined to estimate genotypic differences which might exist between

MMTV genes carried by each of the mouse strains.

The data presented below indicate that (1) all of the mouse strains studied do contain MMTV specific information; (2) virus specific information seems to be integrated into the mouse cell DNA by covalent bonds; (3) BALB/c mice appear to have fewer viral sequences in their DNA than other mouse strains; (4) no significant differences in viral copy numbers could be found between certain "high" (RIII, C3H) and "low" (C3HeB/Fe, C57BL/6) incidence mouse strains; (5) GR mice appear to have two to three times more viral sequences in their genomes than other mouse strains; (6) viral genes present in DNA from various mouse strains have some heterogeneity in nucleotide sequences, (7) genotypic differences exist between the mammary tumor virus released from GR and BALB/c tumor cells and (8) GR cell DNA appears to contain a majority, if not all, viral sequneces for MMTV-P and MMTV-O viruses.

MATERIALS AND METHODS

A. Buffers and Reagents:

1. Deoxyribonuclease - Sigma, deoxyribonuclease I; treated with iodoacetate according to the method of Zimmerman and Sandeen (1966) to remove ribonuclease activity.
2. DNA buffer 0.05 M Tris:HCl, pH, 8.2, 0.01 M EDTA, pH 7, 0.1 M NaCl.
3. G 50 Buffer - 0.6 M NaCl, 0.02 M Tris:HCl, pH 8.2, 0.001 M EDTA.
4. Hydroxylapatite (HAP) - BioRad, Richmond, California, Hydroxylapatite BioGel/HTP DNA grade; BioGel HTP/ column grade.
5. Nonidet-P 40 (NP-40) - Shell Chemical Co., London, England.
6. Phosphate buffer (PB) - equimolar NaH_2PO_4 and Na_2HPO_4, pH 7.
7. Phenol (tris-equilibrated) - Mallinckondt, Reagent grade; 2/3 volume phenol equilibrated with 1/3 volume 0.02 M Tris:HCl, pH 8.2.
8. Pronase (self-digested) - Cal Biochemicals; dissolved in 0.02 M Tris:HCl, pH 7.2 at 10 mg/ml and incubated at 37°C for two hours to digest nucleases.
9. Ribonuclease A (beef pancreatic) - Worthington; dissolved in 0.02 M Tris:HCl, pH 7 at 10 mg/ml and boiled for ten minutes, 100°C, to destroy deoxyribo-

nuclease activity.

10. S1 Buffer - 0.3 M NaCl, 0.003 M $ZnCl_2$, 0.03 M NaAc buffer, pH 4.5, 10 µg/ml heat denatured, sonicated, calf thymus DNA.
11. Sodium dodecyl sulfate (SDS) - Matheson, Coleman and Bell Inc.
12. Sodium acetate buffer (0.1 N, pH 4.5) - 5.6 ml of 1 N Acetic acid, 4.4 ml of 1 N NaAc, and 90 ml of H_2O.
13. SSC - 0.15 M NaCl, 0.015 M sodium citrate, bring to pH 7 with HCl.
14. STE - 0.1 M NaCl, 0.02 M Tris:HCl, pH 7.4, 0.001 M EDTA.
15. TE Buffer - 0.01 M Tris:HCl, pH 7.4, 0.01 M EDTA.

B. Mouse Strains:

Each mouse strain, together with some of its characteristics, has been listed in Table I.

C. Virus:

1. Mouse mammary tumor virus (MMTV)

a. RIII milk virus (MMTV-P (?)) - Mouse mammary tumor virus used in the preparation of MMT viral DNA was kindly supplied by Dr. N. Sarkar. Virus had been purified from the milk of RIII mice according to the procedure of Sarkar and Moore (1968). In this procedure skimmed milk is separated from cream by a low speed centrifugation and clarified by the addition of EDTA. Virus is

TABLE I

Mouse Strains and their General Characteristics

Mouse Strain[1]	Range of tumor incidence in breeding females[4] (%)	Average age of tumor onset in months[4]	Virus strain[5]	Mode of transmission[6]
RIII[2]	60-100	11	MMTV-P (?)	Milk
C3H[3]	80-100	10	MMTV-S	Milk
GR[2]	79-100	7.5	MMTV-P	Milk Gamete
C3HeB/Fe[3,7]	47	19	MMTV-L	Gamete
C57BL/6[3]	1	23.5	(MMTV-Y)	(Gamete)
BALB/c[3]	1	12	MMTV-O	Gamete
	20-30	19		

[1]For the origin and complete history of each strain the reader is referred to: Committee on Standardized Genetic Nomenclature for Mice (1960).

[2]Obtained from colonies maintained in the laboratories of Nural Sarkar, Camden, New Jersey; this colony of mice has 100% incidence of mammary tumors in female mice.

[3]Obtained from Jackson Laboratories, Bar Harbor, Maine.

[4]Ranges reflect the variation in mammary tumor incidence among sublines of each strain housed in different laboratories. Strains RIII, C3H and GR are considered "high" mammary tumor incidence strains because of the occurrence of mammary tumors in almost all breeding females before one year of age. Strains C3HeB/Fe and BALB/c are regarded as "low" mammary tumor indicence strains because few mammary tumors

pelleted from the clarified milk, resuspended and banded by zonal centrifugation on a Ficoll gradient.

b. MMTV-S, MMTV-P, MMTV-O - Mouse mammary tumor virus used for the isolation of MMT virus 70S RNA was obtained from the tissue culture fluids of BALB/c f C3H mammary tumor explants (MMTV-S), GR mammary tumor cell cultures (MMTV-P), or European BALB/c mammary tumor explants by pelleting at 40K for 45 minutes in an SW 41 rotor.

2. Moloney Murine leukemia virus (M-MuLV) - Moloney Murine leukemia virus, isolated from JSL V-9 BALB/c bone marrow cells, was purchased from Electro-Nucleonics Laboratories, Inc., Bethesda, Maryland.

D. Cells:

1. BALB/c - BALB/c cells used in the preparation of "unique sequence" DNA were originally obtained as BALB/c cells from the A31

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develop in these mice before 1.5 to 2 years of age, and then only at a low percentage. Strain C57BL/6 is considered a very low or no incidence strain as few mammary tumors develop in these mice even at old age (Nandi and McGrath, 1973; Schlom et al., 1973).

[5]Virus strains according to the convention of Bentvelzen et al. (1970): S = standard or Bittner agent; P = plaque, RIII and GR strains each have a plaque producing virus but no evidence exists to suggest that they are identical; L = low oncogenic, also know as NIV; Y = yet undiscovered, has not been isolated; O = overlooked.

[6]Information contained within parenthesis is speculative.

[7]Ova transferred from C3H to C57BL/6 by Fekete in 1948: may be considered equal to C3Hf.

clone of Dr. G. Todaro. However, after prolonged passage in this laboratory these cells were no longer highly contact inhibited and grew to a density of $5\text{-}6X10^6/cm^2$. Cells were maintained in Dulbecco's Modified Eagle's Medium (GIBCO), containing 10% calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml, GIBCO).

2. BALB/c f C3H mammary tumor explants - Tissue cultured mammary tumor explants from BALB/c mice foster nursed by C3H mice (BALB/c f C3H) were kindly provided by Dr. S. Nandi and Dr. R. D. Cardiff for the labeling of MMTV-S. Cultures had been prepared as described by McGrath et al. (1972) and were maintained in Eagle's Minimal Essential Media (GIBCO) containing 15% dialyzed fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), insulin (5 µg/ml, Sigma) and hydrocortisone (5 µg/ml, Sigma).

3. European BALB/c mammary tumor cell explants - BALB/c mammary tumors used for the preparation of radioactively labeled MMTV-O virus originally arose spontaneously in a European substrain of BALB/c mice in the laboratory of Dr. P. Bentvelzen. A cell free extract was prepared from the original tumor and injected into newborn BALB/c mice. Mammary tumors developed in a large percentage of the female mice before one year of age. One of these mice was sent to our laboratory where the tumor was subsequently passaged twice as tumor cells in American BALB/c mice before it was removed into tissue culture. The tissue cultures were prepared as described by McGrath et al. (1972) and were maintained in Eagle's MEM containing 15% fetal calf serum, penicillin (100 units/ml),

streptomycin (100 µg/ml), gentamycin (100 µg/ml), insulin (10 µg/ml) and hydrocortisone (10 µg/ml). The concentration of fetal calf serum was reduced to 5% when cultures became confluent.

4. Leydig tumor cells - Leydig tumor cells used for the production of Gross MuLV were a gift of Dr. N. Sarkar. This cell line was originally isolated from an estrogen induced testicular tumor in a (BALB/c X A) X F_1 mouse. In vivo Leydig tumor cells characteristically contain large numbers of intracellular A-particles (Pourreau-Schneider et al., 1968), MMT virus antigens (Nowinski et al., 1971) and large amounts of MMTV specific RNA (Varmus et al., 1973b). However, after prolonged growth in tissue culture, A-particles could no longer be found. When the cells were grown in the presence of cortisol, they began to produce C-particles containing the group-specific antigens of Murine leukemia virus (Sarkar, N., personal communication). B-particles have never been found in Leydig cell tumors or in Leydig tumor cell cultures, and the cells in culture are free of MMT virus-specific RNA (Quintrell and Varmus, unpublished observation).

5. GR mammary tumor cells - GR cells were originally isolated from a GR mouse mammary tumor and propagated in serial culture by Dr. E. Y. Lasfargues. Cells were maintained in our laboratory with Medium 199 containing 10% fetal calf serum, insulin (10 µg/ml), penicillin (100 units/ml) and streptomycin (100 µg/ml).

E. Extraction of Deoxyribonucleic Acid from Mouse Tissues:

Adult male mice were starved for twenty-four hours before death

to reduce liver glycogen. The testis and all internal organs (except the stomach and intestines) were removed as a source of tissue for DNA extraction. DNA was also extracted from whole newborn to one week old RIII and GR mice. Newborn mice were removed from their cages and frozen at -70°C until enough were collected for processing. They were not separated by sex.

Fresh or frozen tissue was homogenized for five minutes in a Waring blender containing ten milliliters of DNA buffer for each gram of tissue. Homogenates were treated with SDS (0.5 %) and pronase (100 µg/ml) overnight at 37°C with constant stirring. The SDS concentration was then increased to a total of 1% and nucleic acids were separated from protein by two phenol extractions at room temperature. An equal volume of phenol (equilibrated with 0.02 M Tris:HCl, pH 8.2) was added to each homogenate, shaken vigorously for ten minutes and centrifuged (10 minutes, 4°C, 2000 r.p.m., in a Sorvall RC 3). Following phenol extraction, nucleic acids were precipitated overnight at -20°C by the addition of two volumes of cold 100% ethanol. The precipitate was collected by centrifugation (20 minutes, 10,000 r.p.m., 4°C, in a Sorvall RC 2B, HB-4 rotor) and resuspended in T.E. buffer (100 ml/40 gm tissue). Resuspended nucleic acid was treated overnight at 37°C with ribonuclease (100 µg/ml) and phenol extracted twice at room temperature without the addition of SDS. To remove phenol and free nucleotides, the DNA solution was dialyzed against 20 volumes of 0.1 X SSC with two changes daily for three days. A one to ten dilution was made of the

dialyzyed DNA and the optical density at 260 nm and 280 nm was determined on a Gilford Model 2000 spectrophotometer. Samples with an O.D. 260/280 ratio of 1.79 or less were re-extracted with phenol and re-dialyzed. The O.D. 260/280 ratio from ten extractions ranged from 1.79-1.93.

Using an extinction coefficient of 45 µg/O.D. unit, the DNA concentration was adjusted to less than 700 µg/ml and sheared at 45-50K p.s.i. in a pressure device designed by the American Instrument Co., Silver Spring, Maryland. DNA sheared in this way is approximately 150-200 nucleotides long (Varmus et al., 1972b; Stavnezer, J., unpublished observation). Sheared DNA was extracted once at room temperature with chloroform-isoamyl alcohol (24:1) and precipitated overnight by the addition of NaAc to 0.2 M and two volumes of cold 100% ethanol. Precipitated DNA was centrifuged at 10,000 r.p.m. and resuspended in 3 mM EDTA.

BALB/c and GR DNA used in hybridization reactions with MMTV-P and and MMTV-O RNA was not sheared mechanically. Instead, it was hydrolyzed by boiling in 0.3 M NaOH for twenty minutes. The DNA solution was then neutralized with HCl and the nucleic acid was precipitated by the addition of 2 volumes of 100% ethanol. DNA sheared by this method varies from approximately 150 to 720 nucleotides in length and has an average length of 360 nucleotides (Stavnezer and Stavnezer, unpublished observations). Shearing by alkaline hydrolysis has certain advantages over mechanical techniques: (a) it is much faster, 100 mg of DNA can be sheared in twenty minutes by alkaline hydrolysis, whereas mechanical shearing would

require several hours of continuous labor; (b) any RNA not digested by treatment with ribonuclease is completely hydrolyzed; (c) DNA can be used in reactions with RNA without further processing since the alkali treatment inactivates ribonuclease. The only advantage of mechanical shearing is that the DNA fragments obtained by this technique are more uniform in size.

Early preparations of mouse cell DNA were chromatographed on Sephadex G50 to remove free nucleotides or small oligonucleotides which might have remained after dialysis. Such nucleotides would increase the optical density of the DNA solution leading to an over estimation of DNA concentration and error in the calculation of Cot values. Fifty to 175 mg of DNA were passed over a 50 cu.cm G50 column and washed with G50 buffer (1 ml/min.). The first peak of absorbance at 260 nm was pooled, ethanol precipitated, centrifuged at 10,000 rpm, resuspended in 3 mM EDTA and the concentration determined by optical density at 260 nm. A later comparative study showed that dialysis alone adequately removed free nucleotides as DNA reannealed similarly at the same Cot values whether or not it had been chromatographed on G50.

Average yields of DNA were 6 mg/adult mouse and 2 mg/gm of newborn mouse tissue. Stocks of purified DNA were stored at -20°C.

F. Preparation of Fractionated Mouse Deoxyribonucleic Acid:

1. "High molecular weight" DNA - Purified "high molecular weight" DNA from ASV virus (B77-C) transformed BALB/c 3T3 cells was provided

by Dr. H. E. Varmus. Cells had been lysed with SDS and centrifuged in a zonal rotor through a 5-40% neutral sucrose gradient by Dr. M. A. Martin. Fractions 2-15 from the bottom of the gradient (52-65S) had been pooled and extracted for DNA (Varmus et al., 1973a).

2. "Network" DNA - "Network" DNA was also provided by Dr. H. E. Varmus. Large molecular weight DNA (84S by analysis in an alkaline sucrose gradient) had been gently extracted from ASV (B77-C) transformed 3T3 cells, heat denatured and reannealed to low Cot values (5 mol-sec/l), which allowed reannealing of repeated but not unique sequences. Under these conditions repeated sequences present on several DNA strands reassociate to form large "networks" which can be centrifuged out of solution (Britten et al., 1965; Varmus et al., 1973c).

3. Nuclear chromatin DNA - Euchromatin, heterochromatin, intermediate and total nuclear chromatin fractions from NIH Swiss mice were a gift of Dr. J. J. Yunis. Chromatin fractionation had been performed by differential centrifugation (Yasmineh and Yunis, 1970). DNA from all fractions was extracted and sheared as described in section E.

4. Preparation of "unique sequence" [C^{14}] deoxyribonucleic acid - BALB/c cells ($2X10^6$ cells/100 mm petri dish) were grown in maintenance medium containing 1 µCi/ml [C^{14}] thymidine (Schwarz/Mann) for 48 hours at 37°C. Cultures were supplemented with fresh serum and incubated for an additional 24 hours, washed twice with DNA buffer, incubated for five minutes at room temperature in DNA buffer

0.5% SDS, scraped with a rubber policeman and processed for DNA as with mouse tissue.

Purified DNA was sheared (50K psi), denatured at 100°C, 3 minutes, reannealed at 69°C in 0.6 M NaCl to a Cot of $2.5X10^2$ mol-sec/l, diluted into 0.01 M PB and separated into single and double stranded DNA by batch elution on hydroxylapatite (0.16 M PB and 0.4 M PB). Forty-two percent of the DNA eluted with 0.4 M PB and represented reassociated, highly redundant, satellite and intermediate families of gene sequences repeated from 100 to 100,000 times. Unhybridized DNA, eluted with 0.16 M PB, presumably represented DNA squences occurring uniquely in the cell genome - "unique sequence" DNA (Britten and Kohne, 1968). The "unique sequence" DNA was dialyzed against 0.1XSSC followed by 0.5 mM EDTA and reduced in volume by flash evaporation. The specific activity of [C^{14}] DNA (determined by optical density (260 nm) and acid precipitable cpm) was $1.45X10^4$ cpm/µgm.

G. Extraction of RNA from Mammary Tumor Tissue:

C3H mammary tumors were homogenized with a Virtis blender for one to two minutes in 0.01 M EDTA, 0.05 M Tris:HCl, pH 9.2, and 0.5% sodium dodecyl sulfate. Homogenates were extracted two to three times at room temperature with phenol which had been equilibrated with 0.05 M Tris:HCl, pH 9.2. Following precipitation with 100% ethanol, the preparation was treated with deoxyribonuclease (20 µg/ml, two hours, room temperature in 0.02 M Tris:HCl, pH 7.4, 0.01 M $MgCl_2$),

phenol extracted twice again and precipitated with 100% ethanol. The precipitate was resuspended in 0.02 M Tris:HCl, pH 7.4, the concentration determined by optical density (260 nM) and stored at -20°C.

H. Synthesis of Viral Deoxyribonucleic Acid:

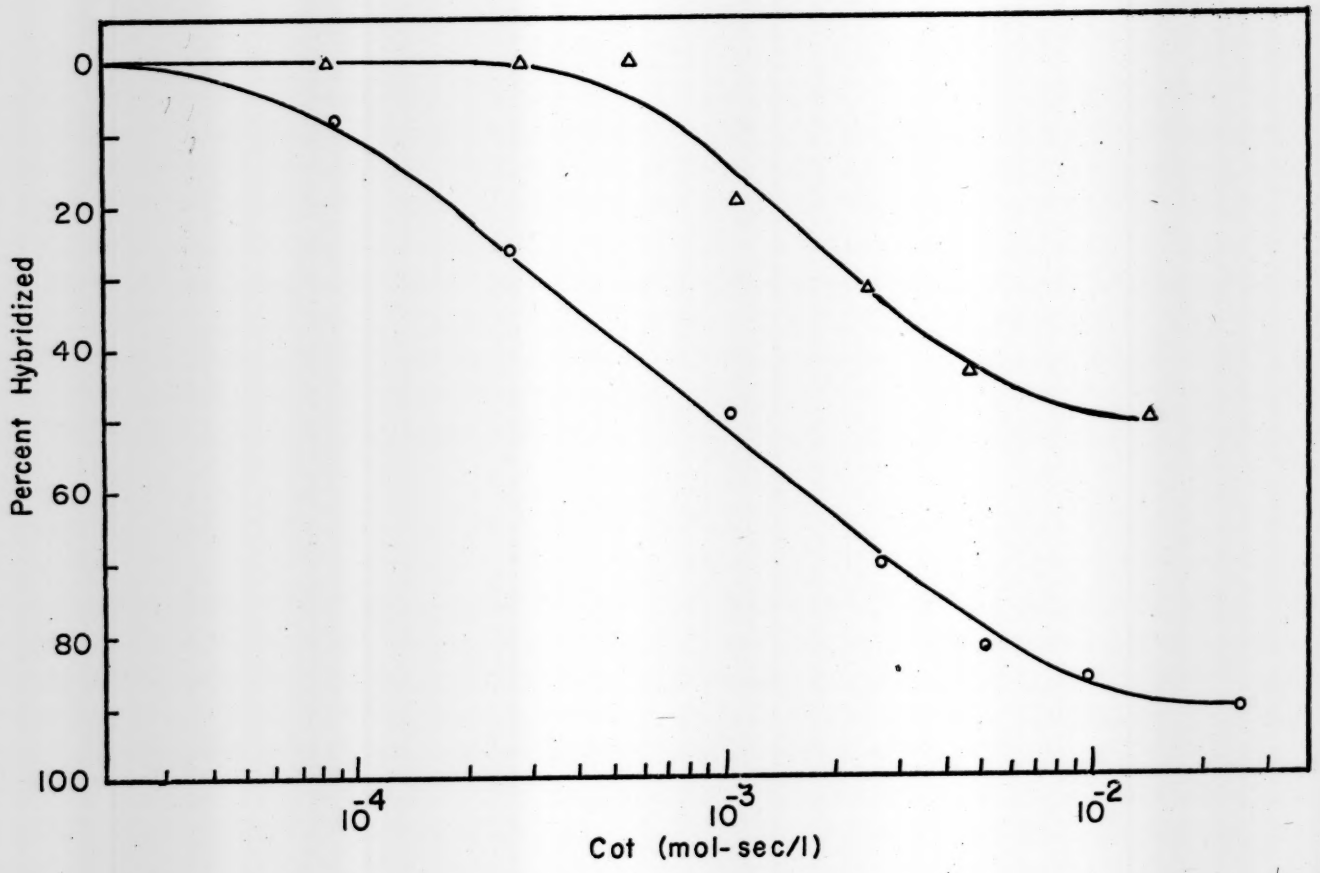
1. Mouse mammary tumor viral double stranded (DS) DNA - MMT viral DS-DNA was synthesized from purified RIII milk MMT virus by an endogenous RNA directed DNA - DNA directed DNA polymerase reaction as described by Varmus et al. (1972a). Reactions contained:

1.2-1.4 mg protein/ml	RIII virus
0.1 M	Tris:HCl, pH 8.2
1.6 mM	$MgCl_2$
1.5% (v/v)	Beta-mercaptoethanol (BME)
0.33% (v/v)	NP 40
$5X10^{-5}M$	[^{3}H]deoxyadenosine triphosphate (dATP), (8.5-18.7 Ci/mM, Schwarz/Mann)
$5X10^{-5}M$	[^{3}H]deoxycytidine tryphosphate (dCTP), (20-30 Ci/mM, Schwarz/Mann)
$5X10^{-5}M$	[^{3}H]deoxyguanosine triphosphate (dGTP), (9.8-20 Ci/mM, Schwarz/Mann)
$5X10^{-5}M$	[^{3}H]thymidine triphosphate (dTTP), (13.4-57 Ci/mM, Schwarz/Mann)

Reaction mixtures were incubated for 18 hours at 37°C and were terminated by the addition of 0.5% SDS. Mixtures were treated with pronase (500 µg/ml, 37°C, 1 hour), followed by two phenol extractions

Figure 1. The reassociation kinetics of MMTV DS-DNA.

MMTV [^{3}H]DS-DNA (2.34×10^{7} cpm/µg, 1.9×10^{-5} mg/ml) prepared from endogenous polymerase reactions was denatured at 100°C then reannealed in 0.4 M phosphate buffer at 68°C (Δ). Samples were removed at increasing time intervals, diluted in 0.01 M PB and assayed by batch elution from hydroxylapatite. To remove non-reassociating DNA, the DS-DNA from endogenous reactions was denatured at 100°C, incubated to a Cot of 10^{-2} mol-sec/l and fractionated on hydroxylapatite. The fraction eluting in 0.4 M PB was then heat denatured, reassociated and assayed as above (o). The percent hybridized is plotted against Cot (mol-sec/l) on a semi-log scale.



at room temperature. Nucleic acids were precipitated in the presence of 200 µg HeLa ribosomal RNA by the addition of 0.2 M NaAc and two volumes of cold 100% ethanol. Precipitates were collected by centrifugation at 10,000 r.p.m. for 30 minutes in a Sorvall RC-2B. Pellets were resuspended in 2 ml of 3 mM EDTA and treated with ribonuclease (100 µg/ml, 1 hr, 37°C). Ribonuclease treated nucleic acids were separated into single and double stranded DNA by batch elution from HAP with 0.18 M and 0.4 M phosphate buffer. The percentage of double stranded DNA varied from 22-62% and could be raised to 75% by increasing the concentration of deoxynucleotide triphosphates to greater than 10^{-4}M. However, such high concentrations of all four [^{3}H]deoxyribonucleotide triphosphates was not practical. Eluents were dialyzed overnight alone or in the presence of 1-50 µg of salmon sperm DNA against 0.5 mM EDTA. Dialyzed viral DNA was concentrated and reassociated as described below.

When MMT viral [^{3}H]DS-DNA was heat denatured, reannealed at 68°C in 0.4 M PB and assayed by HAP, only 50% reassociated at Cot values of 10^{-1} or greater (Figure 1). Varmus et al. (1972a) reported identical results and attributed the non-annealing DNA to thermal scission of minus-strand DNA tails since it hybridized completely to 70S MMTV RNA. Following their procedure, DS-DNA was routinely denatured, reannealed to a Cot of $2X10^{-2}$ mol-sec/l and fractionated on HAP. DNA eluting with 0.4M PB could be reassociated at least 90% by HAP assay and was used in all experiments employing MMTV DS-DNA. The

specific activities for several preparations of DS-DNA ranged from 2.24-3.1X10^{7} cpm/µg (see Appendix for calculations of specific activity).

MMTV double stranded DNA labeled with [^{32}P]deoxyadenosine triphosphate (5X10^{-5}M) was prepared as outlined above using a concentration of unlabeled deoxyribonucleotide triphosphates of 5X10^{-4}M. The specific activity of DS-DNA prepared in this way was estimated to be 2.5X10^{5} cpm/µg (see appendix for calculation).

2. Mouse mammary tumor viral single stranded complementary DNA (cDNA) - MMTV cDNA was synthesized from purified RIII milk MMT virus by an endogenous polymerase reaction as in the synthesis of DS-DNA. For the preparation of MMTV [^{3}H]cDNA the concentration of all four [^{3}H]deoxynucleoside triphosphates was 2.5X10^{-5}M, and actinomycin D (100 µg/ml, Cal Biochemistry) was added to inhibit synthesis of double stranded DNA (McDonnell et al., 1970; Garapin et al., 1973). Reaction mixtures were incubated for 18 hrs. at 37°C and nucleic acids isolated as described above. The percentage of single stranded DNA eluting from HAP in 0.18 M PB varied from 82-97%. When MMTV [^{3}H]cDNA, isolated from HAP, was treated with the single stranded specific nuclease of Aspergillus oryzae (S1), it was hydrolyzed from 96-100% indicating that very little if any double stranded nucleic acid was present.

To inactivate ribonuclease, MMTV cDNA was treated with 0.3 M NaOH for 18 hours, 37°C, neutralized with HCl and precipitated by the addition of two volumes of 100% ethanol. Precipitated cDNA was

centrifuged at 10,000 rpm for 30 minutes, resuspended in 3 mM EDTA and stored at -20°C. The specific activity of viral cDNA ranged from $1.6\text{-}3X10^{7}$ cpm/μgm.

MMTV cDNA labeled with [^{32}P]deoxyadenosine triphosphate was synthesized and isolated as outlined above using a concentration of $2.5X10^{-5}$M unlabeled deoxyribonucleoside triphosphates. The specific activity of this cDNA was estimated as $2.5X10^{5}$ cpm/μg.

3. Murine leukemia virus single stranded complementary DNA - Murine leukemia viral single stranded DNA was synthesized with Moloney MuLV in a reaction which utilized endogenous reverse polymerase activity. Reaction mixtures contained the following:

0.2-1 mg/ml protein	Moloney MuLV
0.1 M	Tris:HCl, pH 8.1
2 mM	$MnCl_2$
0.01% (v/v)	NP 40
1.5% (v/v)	BME
$1X10^{-4}$M	dCTP, dGTP, TTP
$5X10^{-6}$M	dATP
$2.3X10^{-10}$M	[^{32}P]dATP (28.6 Ci/mM)

The same reaction conditions and isolation procedures were used as in the preparation of MMTV cDNA. In addition MuLV cDNA was chromatographed on Sephadex G50 to remove salts which might have precipitated with the nucleic acid. The first peak of radioactivity eluting from the column was ethanol precipitated and resuspended in

3 mM EDTA. The specific activity of Moloney MuLV [^{32}P]cDNA was calculated to be 2.5X10^{5} cpm/µg.

I. Isolation of Radioactively Labeled Viral 70S RNA:

1. MMTV-S 70S RNA - Virus producing cultures of BALB/c f C3H mammary tumor cells were fed with growth media containing 200 µC/ml [^{3}H]cytosine (26 Ci/mM; Schwarz/Mann), [^{3}H]uridine (24.4 Ci/mM; Schwarz/Mann), and [^{3}H]adenosine (18.3 Ci/mM; New England Nuclear Corp.). Labeled media was changed every twelve hours for three days; the first harvest was discarded. Media containing labeled virus was stored at -70°C until further processing.

BALB/c f C3H mammary tumor cultures used for labeling with [^{32}P]orthophosphate (carrier free, New England Nuclear Corp.) were washed three times with special "phosphate free" Medium 199 (GIBCO) containing 10% dialyzed fetal calf serum, insulin (10 µg/ml), cortisol (10 µg/ml, Sigma), penicillin (100 units/ml) and streptomycin (100 µg/ml). After three hours incubation, 37°C, unlabeled "phosphate free" medium was replaced with special "phosphate free" Medium 199 containing 1 mCi/ml [^{32}P]orthophosphate. Labeled cultures were incubated for 12 hours, harvested and relabeled for another 12 hours. Media from the first harvest was discarded and media from the second was processed for viral RNA.

Culture fluid from labeled cells was clarified at 8,500 rpm for 10 minutes, 4°C, in a Sorvall RC 2B. Virus was pelleted from this media (40,000 rpm, 45 minutes, 4°C, SW 41 rotor) and resuspended in 0.5-1 ml STE. A ten minute preincubation with pronase at 37°

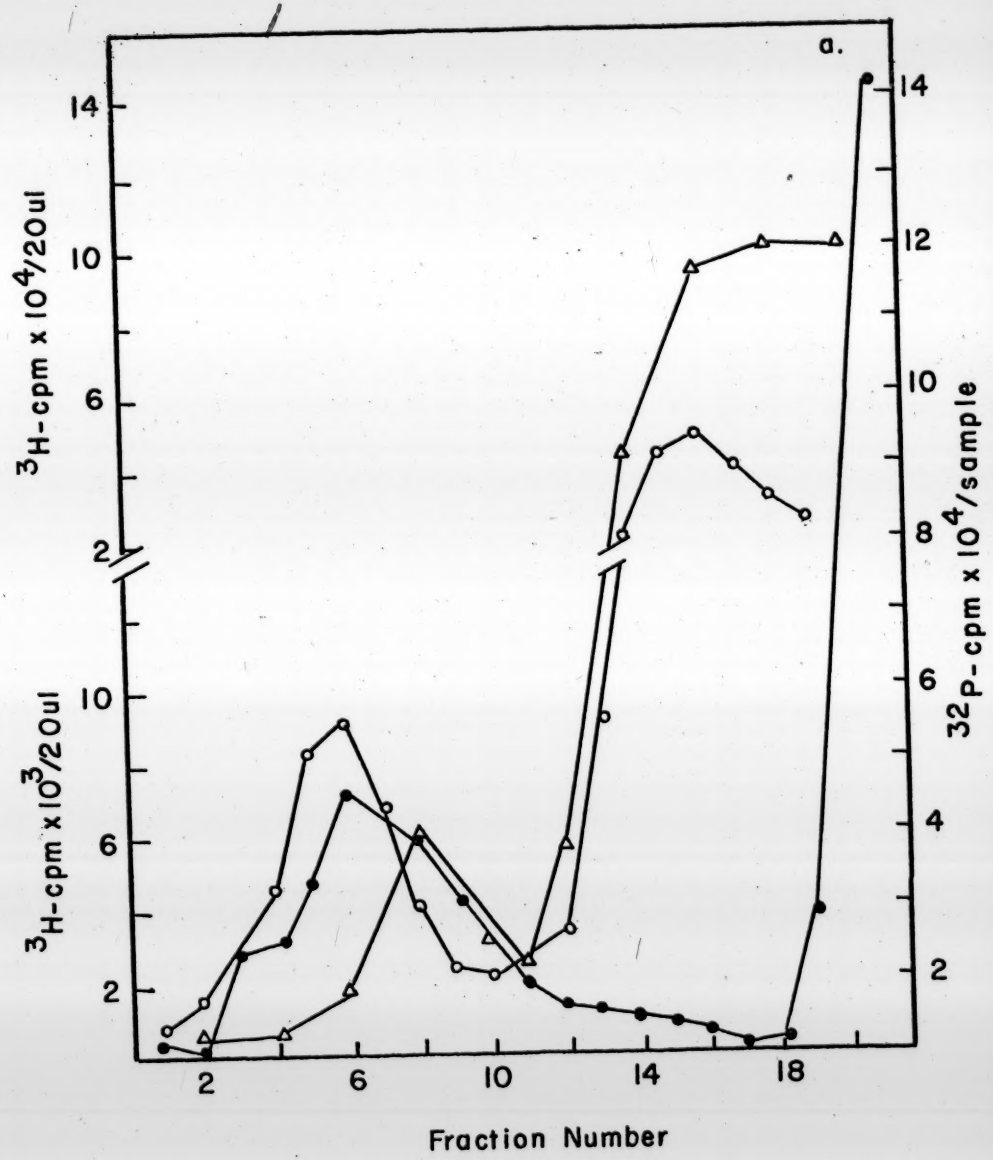
(500 µg/ml) was followed by a thirty minute incubation at 37° after the addition of 0.5% SDS. Virus treated with SDS and pronase was layered onto a preformed 15-30% STE-sucrose gradient and centrifuged at 40,000 rpm for three hours, 4°C in an SW 41 rotor. Gradients were collected through a puncture made in the bottom of each tube and assayed for radioactivity by counting samples in EGME or by measuring Cerenkov radition. Gradient profiles are shown in Figure 2a. The peak of radioactivity in the 70S region of the gradient was pooled and ethanol precipitated at -20°C overnight. RNA precipitates were centrifuged (10,000 rpm, 30 minutes, 4°C), resuspended in 0.01 M Tris:HCl, pH 7.4 and stored at -70°C.

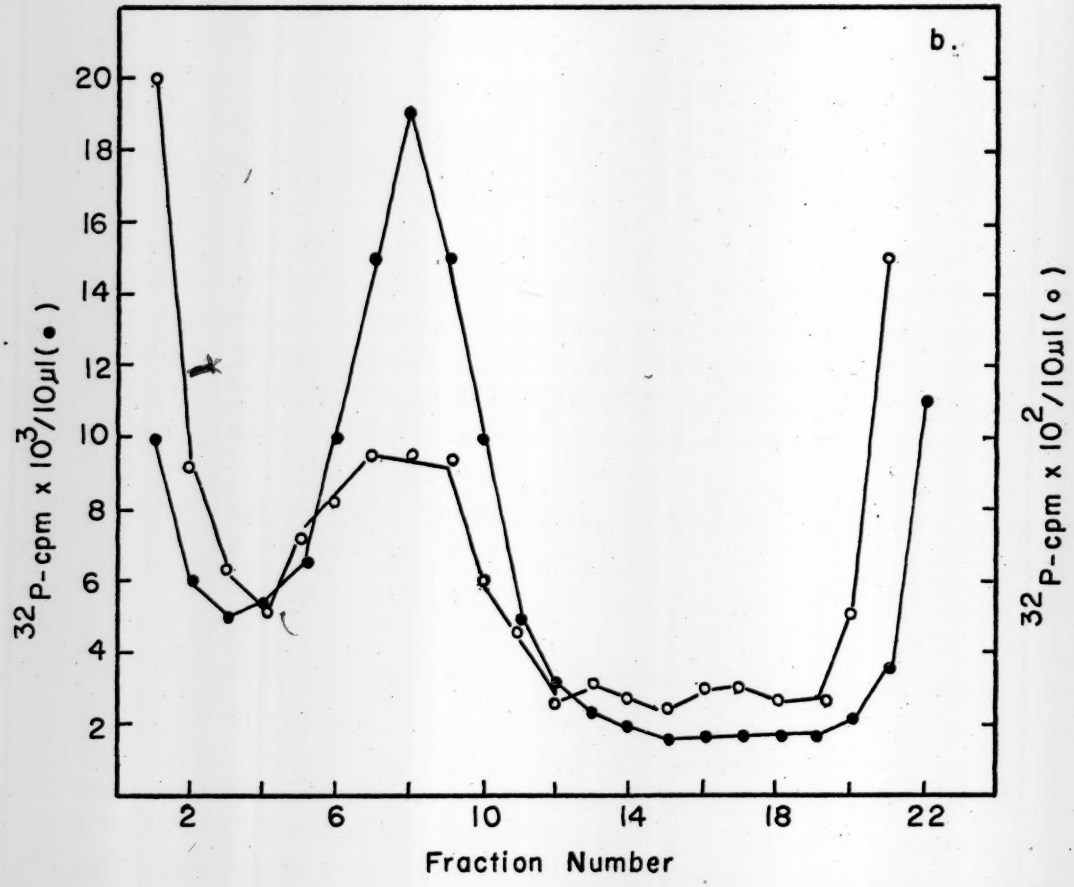
The [^{32}P]labeled material isolated above was from 30-47% resistant to digestion by pancreatic ribonuclease (50 µg/ml, 30 min, 37°C, in 0.1 M NaCl, 0.02 M Tris:HCl, pH 7.4, 0.001 M EDTA), suggesting that it contained significant quantities of macromolecules other than RNA. Resistance to ribonuclease digestion was reduced to 10.3% by treatment with deoxyribonuclease (20 µg/ml 2 hours, room temperature, in 0.02 M Tris:HCl, pH 7.4, 0.01 M $MgCl_2$), followed by two room temperature phenol extractions. Therefore, [^{32}P]labeled DNA, lipoprotein or phospholipids might have also banded in the 70S region on the sucrose gradients. MMTV-S 70S [^{3}H]RNA was 4.5-14.7% ribonuclease resistant under the same conditions of digestion and was not treated further.

The specific activity of viral RNA could not be determined directly because of the minute quantities of viral RNA recovered from mammary tumor explants (approximately 0.2 µg); therefore, the

Figure 2. The isolation of viral 70S RNA by sucrose density gradient centrifugation.

Radioactively labeled virus was isolated from the media of labeled cell cultures by pelleting at 40K for 45 minutes in an SW 41 rotor. Virus pellets were resuspended in 0.5-1 ml STE, treated with 0.5% SDS and 500 µg/ml pronase (37°C, 30 minutes) and (a) layered onto preformed 15-30% STE-sucrose gradients and centrifuged at 40K (3 hr, 4°C, SW41), or (b) layered onto preformed 15-30% STE-sucrose gradients containing 0.1% SDS and centrifuged at 40K (2.5 hr, 22°C, SW41). Gradients were collected from the bottom and assayed for radioactivity. Counts per minute are plotted against fraction number.
(a) Acid-precipitable [^{3}H]cpm/20 µl for [^{3}H]RNA from BALB/c f C3H mammary tumor explants (o); [^{3}H]cpm/20 µl counted in EGME for [^{3}H]RNA from Leydig cell cultures (Δ); and [^{32}P] cpm/fraction, counted by measuring Cerenkov radiation for [^{32}P]RNA from BALB/c f C3H mammary tumor explants (●).
(b) [^{32}P]cpm/10 µl counted by measuring Cerenkov radiation for [^{32}P]RNA from GR cell cultures (●) or European BALB/c mammary tumor explants (o).


a.
^{3}H-cpm x 10^4/20ul
^{3}H-cpm x 10^3/20ul
^{32}P-cpm x 10^4/sample
Fraction Number
14
10
6
2
12
8
4
18


b.
20
18
16
14
12
10
8
6
4
2
^{32}P-cpm x 10^3/10µl(•)
^{32}P-cpm x 10^2/10µl(○)
2
6
10
14
18
22
Fraction Number

specific activity of viral RNA was estimated to equal the specific activity of cellular RNA extracted from the same tissue culture cells used to produce each type of virus. The estimated specific activity was $4.5X10^5$ cpm/µg for MMTV-S 70S [^{3}H]RNA and $4.15X10^6$ cpm/µg for MMTV-S 70S [^{32}P]RNA.

2. MMTV-P and MMTV-0 70S [^{32}P]RNA - GR cell cultures and European BALB/c mammary tumor explants used for the production of MMTV-P and MMTV-0 70S RNA were washed three times and were grown for 12 hours in "phosphate free" Medium 199 containing 10% fetal calf serum, insulin (10 µg/ml) and gentamycin (100 µg/ml). Culture media was replaced every twelve hours thereafter for three days with "phosphate free" Media containing 2 mCi/ml [^{32}P]orthophosphate. Media from the first harvest was discarded. Culture fluids were clarified, virus pelleted, resuspended and treated with SDS and pronase as outlined above. Virus treated with SDS and pronase was layered onto preformed 15-30% STE-sucrose gradients containing 0.1% SDS and centrifuged at 40K rpm for two and a half hours, 22°C in an SW 41 rotor. Gradients were collected from the bottom by inserting a capillary collection tube through the gradient and samples were assayed for radioactivity by measuring Cerenkov radiation. Gradient profiles are depicted in Figure 2b. The 70S peak of radioactivity was pooled, treated with pronase (500 µg/ml, 45 minutes, 37°C), extracted twice at room temperature with phenol, ethanol precipitated in the presence of 100 µg yeast RNA, resuspended in a small volume of 0.02 M Tris:HCl, pH 7.4 and stored at -70°C. MMTV-P

70S [^{32}P]RNA prepared in this way from dexamethasone stimulated GR cell cultures was 10-14% resistant to digestion by pancreatic ribonuclease in 2XSSC for 30 minutes, 37°C. MMTV-O 70S [^{32}P]RNA prepared from European BALB/c mammary tumor explants was 12% resistant to digestion by pancreatic ribonuclease under similar conditions of digestion. The specific activity of these preparations was estimated as being equal to ribosomal RNA extracted from virus producing cells and was $8.7X10^6$ cpm/µg for both types of viral RNA.

3. Murine leukemia virus 70S RNA - Leydig tumor cells producing Gross murine leukemia virus were labeled with growth medium containing 100 µCi/ml of [^{3}H]cytodine, [^{3}H]uridine and [^{3}H]adenosine. Procedures for labeling and for isolation of 70S RNA were the same as those used for MMTV 70S [^{3}H]RNA. The specific activity of MuLV 70S [^{3}H]RNA, $4.5X10^5$ cpm/µg, was estimated as above.

J. Isolation of Radioactively Labeled Cellular RNA:

1. Unfractionated cellular RNA - RNA was extracted from BALB/c f C3H, GR and Leydig cells which had been used for the production of radioactively labeled virus. Cell cultures were incubated for one hour, 37°C, in STE containing SDS (0.5%) and pronase (500 µg/ml), scraped with a rubber policeman and pooled for two phenol extractions, 60°C, 5 minutes, with periodic shaking. A third phenol extraction was carried out at room temperature and nucleic acids were precipitated by the addition of 0.2 M NaAc and two volumes of 100% ethanol. Precipitates were centrifuged (10,000 rpm, 30 minutes, 4°C), resuspended in 0.2 M Tris:HCl, pH 7.4, 0.01 M $MgCl_2$ and

treated for two hours at room temperature with deoxyribonuclease (20 µg/ml). The reaction was terminated with the addition of 0.01 M EDTA, phenol extracted twice (room temperature), ethanol precipitated and resuspended in 0.02 M Tris:HCl, pH 7.4. The RNA concentration was determined by absorbance at 260 nm. Using an extinction coefficient of 40 µg/ml/O.D. 260 nm unit, the specific activity of [^{3}H]RNA from BALB/c f C3H and Leydig cells was $4.5X10^{5}$ cpm/µg, while [^{32}P]RNA from BALB/c f C3H cells was $4.2X10^{6}$ cpm/µg and from GR cells was $8.6X10^{6}$ cpm/µg.

2. Ribosomal RNA - GR mouse mammary tumor cells were labeled with [^{32}P]orthophosphate, 0.17 mCi/ml, twice daily for two days in "phosphate free" Medium 199 containing 4% calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). Total cellular RNA, extracted as outlined, was resuspended in STE and separated on a preformed 15-30% STE-sucrose gradient (28,000 rpm, 15 hours, 4°C, SW 41 rotor). The gradient was collected through a puncture in the bottom of the tube and fractions were assayed by measuring Cerenkov radiations. The 28, 18 and 4S regions of the gradients were separately ethanol-precipitated, resuspended in 0.02 M Tris:HCl, pH 7.4 and concentrations determined by optical density (260 nm). The specific activity of GR ribosomal [^{32}P]RNA was $8.3X10^{5}$ cpm/µg.

K. Nucleic Acid Hybridization:

1. Reassociation of MMTV DS-DNA in the presence of mouse cell DNA - Hybridizations were performed according to the procedures

of Varmus et al. (1972). MMTV [^{3}H]DS-DNA (1.1 ng/ml, 2.6X10^7 cpm/µg) was mixed with one or four milligrams per milliliter of mouse DNA, denatured at 100°C 5 minutes and adjusted to 0.4 M PB. Annealing was carried out in microflex tubes (Kontes) under mineral oil at 68°C. Samples (15 µl/100 µl reaction) were removed at appropriate times into 2 ml of cold 0.01 M PB and stored at 4°C until hybridizations were completed. To follow the reassociation of [^{3}H]DS-DNA under the same conditions of viscosity and temperature, a control reaction containing MMTV [^{3}H]DS-DNA (1.1 ng/ml) and salmon sperm DNA (1 or 4 mg/ml) was included with each set of hybridizations. The extent of hybridization was determined by fractionation with HAP using 0.16 M and 0.4 M PB. By convention (Britten and Kohne, 1968), the fraction hybridized was plotted against the product of the initial DS-DNA concentration and time (Cot). Cot values were corrected to a standard sodium ion concentration of 0.12 M using the tables of Britten and Smith (1970).

2. MMTV cDNA hybridized with mouse cell DNA - MMTV [^{3}H]cDNA (0.32 ng/ml, 2.5-3X10^7 cpm/µg) was mixed with mouse "unique sequence" [^{14}C]DNA (64 ng/ml, 1.45X10^4 cpm/µg) and unlabeled mouse DNA (4 mg/ml) in 5 ml microflex tubes, denatured 3-5 minutes at 100°C, adjusted to 0.6 M NaCl, covered with mineral oil and incubated at 68°C. Samples (0.5 ml) were removed at intervals into 3.5 ml S1 buffer and stored at 4°C. Hybridization was assayed by digestion with S1 nuclease and the percent hybridized was plotted against Cot (corrected to 0.12M Na^+) for unlabeled mouse DNA.

3. MMTV DS-DNA reassociated in the presence of MMTV cDNA - Single and double stranded MMTV DNA were annealed at a cDNA/DS-DNA ratio of 1:62.5. MMTV [^{3}H]cDNA (2.02 ng/ml, 3X10^{7} cpm/µg) and MMTV [^{32}P]DS-DNA (125 ng/ml, 2.5X10^{5} cpm/µg) were mixed, denatured at 100°C for three minutes, adjusted to 0.6 M NaCl or 0.4 M PB, covered with mineral oil and incubated at 68°C. Samples (30 µl/125 µl reaction) taken from reactions containing NaCl were diluted into 4 ml S1 buffer and assayed for resistance to digestion by S1 nuclease. Samples from hybridizations performed in phosphate buffer were diluted into 2 ml of 0.01 M PB and assayed by fractionation on hydroxylapatite (elution with 0.18 M and 0.4 M PB).

4. MMTV cDNA hybridized with C3H mammary tumor RNA - Four preparations of MMTV [^{3}H]cDNA (0.036-0.058 ng, 1.6-3X10^{7} cpm/µg) were hybridized to increasing quantities of C3H mammary tumor RNA (0.14 ng-0.14 mg), in 0.3 M NaCl, 0.025 M Tris:HCl, pH 7.4, 0.001 M EDTA. Fifty microliter reactions were incubated in plastic tubes under paraffin oil at 68°C for 40 hours. Hybridization was assayed by digestion with S1 nuclease and the fraction hybridized was plotted against the product of the RNA concentration and time (Crt) according to the convention adopted by Birnstiel et al. (1972) and Leong et al. (1972). These hybridizations were kindly performed by N. Quintrell.

5. Viral cDNA hybridized with viral 70S RNA - Using a modification of the conditions described by Duesberg and Canaani (1970) and Garapin et al. (1973), MMTV 70S [^{3}H]RNA (2.66 ng, 4.5X10^{5} cpm/µg) was hybridized with increasing amounts of MMTV

[^{32}P]cDNA (1.33 ng to 138 ng, 2.5X10^5 cpm/µg), or Moloney MuLV [P^{32}]cDNA (1.33 ng to 37 ng, 2.5X10^5 cpm/µg). Gross MuLV 70S [^{3}H]RNA (2.92 ng, 4.5X10^5 cpm/µg) was hybridized with increasing amounts of Moloney MuLV [^{32}P]cDNA (1.46 ng to 36.5 ng, 2.5X10^5 cpm/µg). Hybridization was performed in 10 µl or 40 µl reaction volumes, between paraffin oil sealed in microcapillary pipettes, in the presence of HeLa cell RNA (46 ng), 0.6 M or 0.9 M PB, at 68°C for 60-70 hours. HeLa cell RNA was added to compete with 70S RNA for trace amounts of contaminating ribonuclease. Reactions were assayed for resistance to digestion by pancreatic ribonuclease. The hybridization mixtures were expressed into 2 ml of 2XSSC and divided in half. Ribonuclease (50 µg/ml) was added to one of the two aliquots and both were incubated for 30 minutes at 37°C. Following incubation, carrier protein (30 µg bovine serum albumin) was added to tubes not treated with ribonuclease and all samples were precipitated with 5% TCA, filtered, dried and counted in toluene-liquiflur in a Beckman Liquid Scintillation counter. The fraction ribonuclease resistance was calculated and corrected for intrinsic ribonuclease resistance (MMTV 70S [^{3}H]RNA, 4.5-14.5%; MuLV 70S [^{3}H]RNA, 27%).

6. <u>MMTV 70S [^{32}P]RNA hybridized with excessive amounts of mouse DNA</u> - Hybridization conditions are modifications of those described by Melli <u>et al</u>. (1972), Neiman (1972) and Hansen (1972). MMTV 70S [^{32}P]RNA (5 ng, 8.7X10^6 cpm/µg or 6.6 ng, 4.5X10^6 cpm/µg) was mixed with mouse cellular DNA (10 mg/ml) or salmon sperm DNA

(10 mg/ml) in 5 ml microflex tubes, heated to 100°C for 3 minutes, adjusted to 0.4 M PB or 4XSSC, covered with mineral oil and incubated at 68°C. GR cell 18S ribosomal [^{32}P]RNA (10 ng/ml, 8.3X10^{5} cpm/µg), was hybridized to GR DNA (10 mg/ml) under the same conditions. Samples were removed at various times and assayed for resistance to digestion by ribonuclease as described above (K,5) except that no carrier protein was used. The percent ribonuclease resistance was corrected for intrinsic ribonuclease resistance (MMTV 70S RNA: MMTV-S, 11%; MMTV-P, 14%; MMTV-O, 12.25%. Ribosomal 18S RNA, 10%).

L. Hydroxylapatite (HAP) Fractionation of DNA:

1. Batch elution - Fractionation on HAP (DNA grade Bio Gel/HTP, BioRad Laboratories) by batch elution had been described by Fanshier et al. (1972). DNA (60 µg or less) in 0.01 M PB was adsorbed to 1 ml of HAP suspension (10 gm HAP crystals/50 ml of 0.01 M PB) for 15 minutes at room temperature. Adsorbed single stranded DNA was removed from HAP by two successive washes at 68°C with 0.16 M or 0.18 M PB, and double stranded DNA was eluted by two washes with 0.4 M PB. The two washes at each concentration of PB were generally pooled. Hybridization of unlabeled DNA was determined by comparing the absorbance at 260 nm of low and high PB eluants. To assay for hybridization of radioactive nucleic acids, the pooled supernates from each wash were acid precipitated in the presence of calf thymus carrier DNA (80 µg) with 5% TCA, filtered on glass fiber filters

(Reeve-Angel), washed with 5% TCA, 95% ethanol, dried and counted in toluene-liquifluor solution in a Beckman Scintillation counter. The percent nucleic acid hybridized =

$$\frac{\text{acid precipitable cpm in the 0.4 M PB elution}}{\text{total acid precipitable cpm eluted in 0.16 M and 0.4 M PB}} \times 100$$

Because of slight variations in the elution properties of DNA with different lots of HAP or with different preparations of phosphate buffer, nucleic acid standards containing radioactive single or double stranded DNA (approximately 10^3 cpm) and salmon sperm DNA (the same concentration as in experimental samples) were included in each batch analysis. The following corrections were made for variations in DNA elution properties:

$$\frac{\text{(percent hybridized) - (percent single stranded eluting in 0.4 M PB)}}{\text{(percent double stranded control eluting in 0.4 M PB) - (percent single stranded control eluting in 0.4 M PB)}}$$

The corrected percent hybridized was used in all figures.

2. Hydroxylapatite column separation - Hydroxylapatite column separations were performed in 25 ml plastic syringes contained within a temperature controlled water chamber. Hydroxylapatite (10 gm/25 ml of 0.12 M PB, Bio-Gel HTP/column grade) was boiled at 100°C for 10 minutes to eliminate channeling and air bubbles and to increase the flow rate of the columns. HAP solution was allowed to cool at

60°C and poured into syringes (1 ml packed HAP/200 µg added DNA). DNA was applied to columns in 0.12 M PB and eluted with three washes of 0.12 M PB (total volume of 3.6 ml PB/1 ml packed HAP).

M. S1 Nuclease Digestion:

Isolation and conditions of digestion by Aspergillus oryzae S1 nuclease have been reported by Ando (1966) and Leong et al. (1972). Samples containing hybridized material were diluted into 2 ml of S1 buffer and divided into 1 ml aliquots. S1 nuclease was added to one half of the tubes and all aliquots were incubated at 50°C for 2 hours. Nucleic acids were precipitated with 5% TCA, filtered and counted as above (L,1). The fraction of resistance to digestion by S1 nuclease (fraction hybridized) was determined as:

$$\frac{\text{acid precipitable cpm after S1 nuclease digestion}}{\text{acid precipitable cpm with no nuclease digestion}}$$

Corrections for intrinsic S1 resistance of viral cDNA were made as follows:

$$\frac{\text{(fraction S1 nuclease resistance) - (fraction intrinsic S1 resistance)}}{\text{(1.0) - (fraction intrinsic S1 nuclease resistance)}}$$

The corrected fraction hybridized was used in all figures.

N. Thermal Denaturation of Hybridized Viral Nucleic Acids:

1. Thermal denaturation assayed by S1 nuclease digestion - MMTV [^{3}H]DNA (0.153 ng/ml, 2.5X10^7 cpm/µg) was hybridized to 4 mg/ml of mouse cell DNA for 72 hours at 68°C in 0.6 M NaCl. Following hybridization, reactions were ethanol precipitated (2X volumes 100% ethanol, -20°C, overnight), resuspended in 2.2 ml of 0.2 M Tris:HCl, pH 7.3 and divided into 0.2 ml aliquots. Each sample (0.2 ml) was denatured at the desired temperature for 15 minutes and chilled immediately in an ice water bath. Two milliliters of S1 buffer were added to each sample and the percentage of denatured hybrid was determined by digestion with S1 nuclease. MMTV [^{3}H]DS-DNA (1.8X10^4 cpm), reannealed to remove unhybridizable material, was added to 2.2 ml of 0.02 M Tris:HCl, pH 7.3, denatured and assayed as above.

2. Thermal elution from hybroxylapatite - MMTV [^{3}H]cDNA (0.25 ng/ml, 3X10^7 cpm/µg) and "unique sequence" [C^{14}]DNA (216 ng/ml, 1.45X10^4 cpm/µg) were hybridized to 4 mg/ml mouse cell DNA in 0.4 M PB at 68°C for 69-73 hours. Reactions were diluted to 0.12 M PB and hybridized DNA was adsorbed to HAP columns at 60°C. The temperature of the columns was increased by increments of 5°C from 6 to 95°C, allowed to equilibrate for 15 minutes at each temperature, and denatured DNA was eluted with 0.12 M PB. The percent of denatured hybrid was determined by:

$$\frac{\text{acid precipitate cpm eluting at each temperature}}{\text{total acid precipitable cpm eluted from the column}} \times 100$$

The percent denatured at each temperature was added to the percent denatured at lower temperatures to obtain the "cumulative percent denatured DNA".

3. Thermal denaturation of annealed MMTV 70S RNA - MMTV-P (3.07 ng, $2X10^4$ cpm) and MMTV-0 (4.2 ng, $3X10^4$ cpm) 70S [^{32}P]RNA were separately annealed to GR (6.14 mg) or BALB/c (9.2 mg) DNA at 10 mg/ml of DNA in 4 X SSC, at 68°C. Reactions were carried to a Cot of $4X10^6$ mol-sec/l, ethanol precipitated and nucleic acids were resuspended in 0.01 M Tris:HCl, pH 8.1. Samples (0.3 ml) from each reaction were heated for fifteen minutes at various temperatures, cooled immediately in an ice water bath, diluted with 2 ml of 2XSSC and separated into two 1 ml fractions. The extent of denaturation was then assessed by treating one fraction with pancreatic ribonuclease (50 µg/ml). Both fractions were incubated for 30 minutes at 37°C, acid precipitated with an equal volume of 10% TCA, filtered and counted in liquiflur-toluene. The results have been presented as the percent of hybrid denatured at each temperature and have been corrected for the percentage of unannealed [^{32}P]RNA before thermal denaturation. Corrections were as follows: MMTV-P RNA + GR DNA = 22%; MMTV-P RNA + BALB/c DNA = 43%; MMTV-0 DNA + GR DNA = 32%; MMTV-0 RNA + BALB/c DNA = 36%.

RESULTS

A. Detection of Mouse Mammary Tumor Virus Specific Sequences in Mouse Cell DNA by Accelerated Reassociation of MMTV DS-DNA:

1. Enumeration of MMTV specific sequences - When annealing of DNA occurs under fixed conditions (monovalent cation concentration, nucleotide chain length, temperature and viscosity), the reassociation rate for a particular DNA is directly proportional to its concentration (Britten and Kohne, 1968). By the same principle, when viral DS-DNA is reannealed in the presence of another DNA species, any viral sequences present in that species will increase the concentration of viral DNA and accelerate the rate of reassociation of DS-DNA (Gelb et al., 1971b).

Using this premise, Varmus et al. (1972a) compared the effect of DNA from mice with high (GR) and low (C57BL/6) incidence of mammary tumors upon the reassociation of MMTV DS-DNA. They found that DNA from both strains of mice equally accelerated the reassociation rate of viral DNA. These experiments have been repeated here and expanded to include other strains of mice with varying incidences of mammary tumors.

The reassociation of MMTV [^{3}H]DS-DNA in the presence of mouse or salmon sperm DNA (as described in Materials and Methods) is illustrated in Figures 3 and 4. Viral DS-DNA reassociated in the presence of 1 or 4 mg/ml of salmon sperm DNA with the same kinetics as when reannealed alone (Figure 4). The reassociation of MMTV [^{3}H]DS-DNA was significantly accelerated, however, in the presence of RIII,

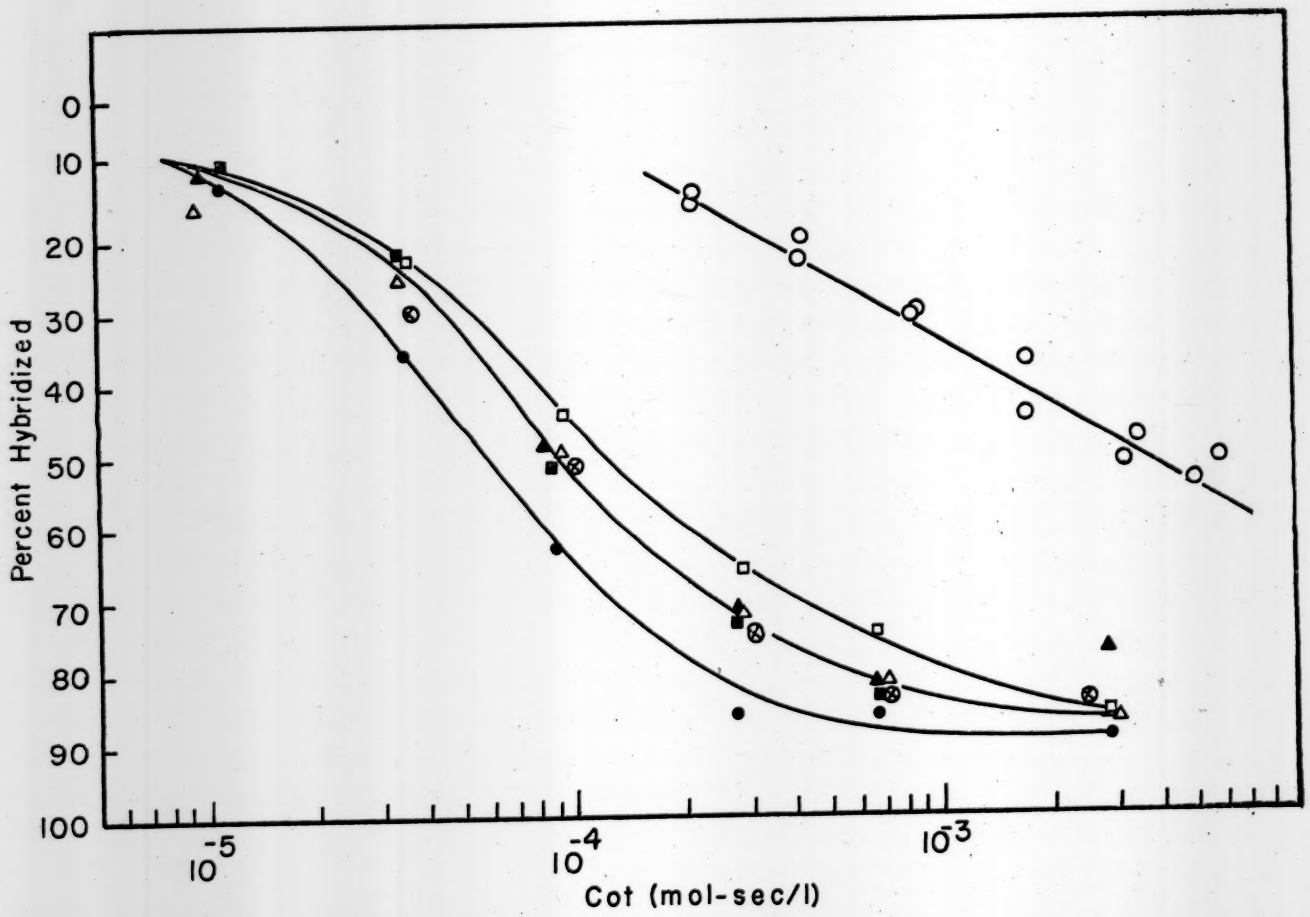
C3H, GR, C3HeB/Fe, C57BL/6 or BALB/c DNA (Figure 3). The reassociation curves closely followed second order kinetics and were compared at their midpoint or half period of reassociation (Cot1/2; Britten and Kohne, 1968). The relative rate of acceleration and the estimated number of viral genome equivalents per mouse cell (copy numbers) are listed in Table II for each mouse strain. Calculations were similar to those used by Gelb, Kohne and Martin (1971) for measurement of SV 40 DNA in infected cells. Details are presented in the Appendix.

The number of viral copies per diploid cell varied from 22 to 45. There appeared to be no correlation between mammary tumor incidence and the number of viral genome equivalents per mouse cell. The high tumor incidence strains GR and RIII did not differ significantly from the low tumor strains C3HeB/Fe, C57BL/6 or BALB/c. Strain C3H did show a 1.5-2 fold increase in its relative rate of acceleration over other mouse strains. But a two fold difference in copy numbers is of uncertain significance as small differences in the reassociation curve can yield large differences in copy numbers, and Varmus et al. (1972a) have reported this much variability within the same mouse strain.

Although the principal finding of no difference in viral copy numbers among high and low mammary tumor incidence strains of mice corresponds with the work of Varmus et al. (1972a), the absolute copy numbers given in Table II are smaller than the previously reported numbers of 60 to 100 for GR and C57BL/6 strains of mice

Figure 3. The effect of mouse cellular DNA on the reassociation of MMTV DS-DNA.

MMTV [^{3}H]DS-DNA (1.26×10^{-6} mg/ml, 2.6×10^{7} cpm/μg) was reannealed in the presence of 4 mg/ml of cellular DNA from the following mouse strains: RIII (□), C3H (●), GR (⊗), C3HeB/Fe (△), C57BL/6 (■), BALB/c (▲), or in the presence of 4 mg/ml of salmon sperm DNA (o). Hybridizations were carried out in 0.4 M PB at 68°C. The percent of reassociation for MMTV DS-DNA was determined by fractionation on hydroxylapatite and plotted as percent hybridized against Cot (mol-sec/l.) on a semi-log scale.


Percent Hybridized
0
10
20
30
40
50
60
70
80
90
100
10^{-5}
10^{-4}
10^{-3}
Cot (mol-sec/l)

(Varmus et al., 1972a). This difference in copy numbers can be explained by the higher Cot 1/2 of reassociation for MMTV DS-DNA in the presence of salmon sperm DNA ($4X10^{-3}$ mol-sec/l) used by Varmus et al. (1972a) when computing viral copy numbers.

TABLE II

The number of MMTV genome equivalents per diploid mouse cell determined by accelerated reassociation of MMTV DS-DNA

DNA	Half period of reassociation (Cotl/2)	Relative rate of acceleration*	Number of viral genome equivalents per diploid cell**
salmon sperm	$2.3X10^{-3}$	0	0
RIII	$9.6X10^{-5}$	24	22
C3H	$4.7X10^{-5}$	49	45
GR	$7.5X10^{-5}$	31	29
C3HeB/Fe	$7.5X10^{-5}$	31	29
C57BL/6	$7.5X10^{-5}$	31	29
BALB/c	$7.5X10^{-5}$	31	29

* $$\frac{\text{(Cotl/2, MMTV } [^3\text{H}]\text{DS-DNA + salmon sperm DNA)}}{\text{(Cotl/2, MMTV } [^3\text{H}]\text{DS-DNA + mouse cell DNA)}}$$

** Copy number based on a complexity of $1.2X10^6$ for MMTV DS-DNA, see Discussion.

2. Integration of mouse mammary tumor virus specific sequences in mouse cell DNA - The relationship of MMTV specific DNA to the mouse cell genome was determined in three ways. First, viral [^{3}H]DS-DNA was reassociated with mouse DNA that had been isolated as "high molecular weight" native DNA (see Materials and Methods). A double stranded DNA copy of the MMTV genome (approximately $2X10^7$ daltons or less) would have to be closely associated with the mouse genome to appear with this DNA (greater than $5X10^7$ daltons) on a sucrose gradient. If viral DNA were not associated with mouse DNA, the "high molecular weight" DNA would be less effective than total cellular DNA in accelerating reassociation. "High molecular weight" DNA was as efficient as total mouse DNA in accelerating the reassociation of MMTV [^{3}H]DS-DNA (Figure 4, Table III). Therefore, viral DNA must be intimately associated with the mouse cell genome.

Secondly, the possibility of mammary tumor virus specific DNA being associated with the mouse cell genome through covalent linkage was examined using the procedure employed by Varmus et al. (1973c) to study the integration of Rous Sarcoma Virus specific DNA. This technique was based on the observation (Britten et al., 1965) that when DNA from higher organisms is heat-denatured and reassociated to low Cot values, multibranched structures of reassociated DNA (networks) form between multiple repeated sequences. These networks can be pelleted by brief centrifugation. Virus specific DNA covalently linked to strands containing reiterated DNA would also be removed in networks, while unintegrated sequences

Figure 4. The effect of fractionated mouse cellular DNA upon the reassociation of MMTV DS-DNA.

MMTV [^{3}H]DS-DNA ($1.1X10^{-6}$ mg/ml, $2.6X10^7$ cpm/µg) was annealed in the presence of 1 or 4 mg/ml (△) of salmon sperm DNA, 4 mg/ml of DNA from B77 Rous sarcoma transformed BALB/c 3T3 cells lysed with SDS and centrifuged through a 5-40% neutral sucrose gradient (◑), 1 mg/ml of "network" DNA from the same cells (■), or 1 mg/ml of NIH Swiss total cellular (▲), euchromatin (o), heterochromatin (□), or intermediate (●) nuclear DNA. (See Materials and Methods for the preparation of DNA fractions.) MMTV [^{3}H]DS-DNA (0.014 µg/ml, $2.6X10^6$ cpm/µg) was denatured and reassociated without additional DNA (⊗). Hybridization was performed and assayed as in Figure 3.

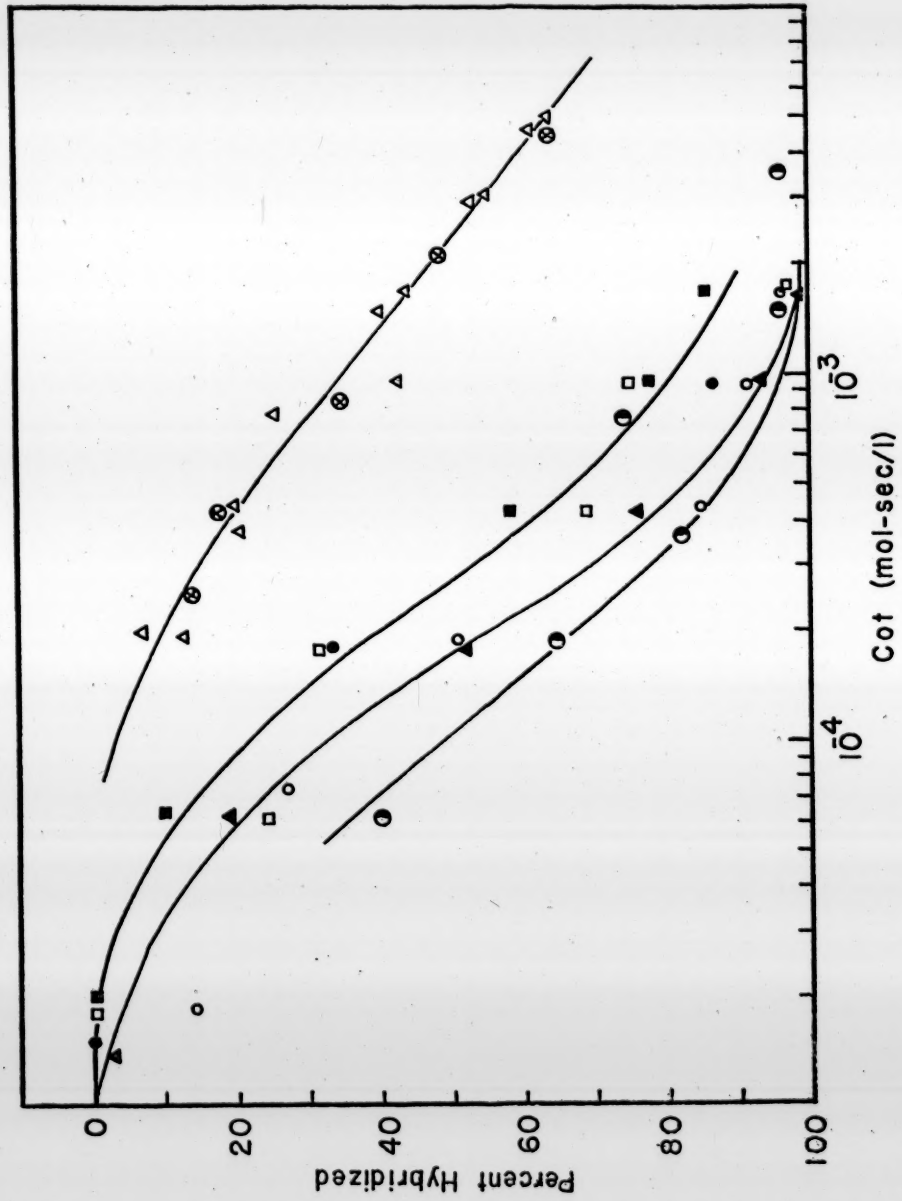

Percent Hybridized
0
20
40
60
80
100
10^{-4}
10^{-3}
Cot (mol-sec/l)

TABLE III

The number of MMTV genome equivalents in mouse cell DNA fractions as determined by accelerated reassociation of MMTV DS-DNA*

DNA	Half period of reassociation (Cot1/2)	Relative rate of acceleration	Number of viral genome equivalents per diploid mouse cell
salmon sperm	$2.3X10^{-3}$	0	0
"high molecular weight DNA"	$1.05X10^{-4}$	22	20
network DNA	$2.7X10^{-4}$	8.5	31
NIH Swiss chromatin fractions			
total nuclear DNA	$1.7X10^{-4}$	13.5	50
hetero-chromatin	$2.7X10^{-4}$	8.5	31
intermediate chromatin	$2.7X10^{-4}$	8.5	31
euchromatin	$1.7X10^{-4}$	13.5	50

* Data are taken from Figure 4.

would remain in the supernatant fraction.

When RSV-transformed BALB/c 3T3 high molecular weight DNA was reannealed to a Cot of 5 mol-sec/l, 95% of the DNA was recovered as networks (networks were prepared by Dr. H. E. Varmus). Sheared network DNA was denatured and reannealed in the presence of MMTV [^{3}H]DS-DNA to assess its ability to accelerate the reassociation of viral DNA. Reassociation was assayed by fractionation of HAP and the results are presented in Figure 4. Network DNA (Table III) was as efficient as total mouse cellular DNA (Table II) in accelerating the reassociation of viral [^{3}H]DS-DNA, indicating that virus specific DNA is covalently linked to cellular DNA containing repeated sequences.

Neither of the above experiments entirely eliminates the possibility that multiple viral DNA genomes might be linked in tandem and not covalently attached to cellular DNA. Such tandemly linked viral DNA could appear with "high molecular weight" DNA on a sucrose gradient. Although the low Cot value of 5 mol-sec/l used in making networks should not be sufficient to allow the reassociation of viral sequences (even if they were reiterated as many as 135 times per diploid cell), and control experiments have shown that only 10% of the lambda phage included with "high molecular weight" DNA was trapped in the pellet during network formation (Varmus et al., 1972b), the trapping or pelleting of MMTV DNA during network formation remains a possibility.

Thirdly, mouse cell nuclear chromatin fractions were tested for

enrichment of MMTV sequences by their ability to accelerate the reassociation of MMTV DS-DNA. Chromatin from NIH Swiss mice was fractionated by Dr. J. Yunis, using differential centrifugation and precipitation properties of sonicated nuclear chromatin (Yasmineh and Yunis, 1970). This method of isolation yields three chromatin fractions: 1) euchromatin (non-condensed chromatin), which represents 82% of the total nuclear DNA and acts as a template for the transcription of most messenger RNA; 2) heterochromatin (condensed chromatin), which represents approximately 10% of the total nuclear DNA and is composed primarily of satellite DNA; and 3) intermediate chromatin, which comprises the remaining 8% of nuclear DNA and is a mixture of euchromatin and heterochromatin (Yasmineh and Yunis, 1970). If virus specific DNA were only integrated into one of these chromatin fractions, that fraction would be enriched in viral sequences and would more markedly accelerate the reassociation of viral DS-DNA than would total nuclear DNA; fractions not containing virus-specific sequences should not affect the rate of reassociation. Results for the reassociation of MMTV [^{3}H]DS-DNA in the presence of DNA from each chromatin fraction are illustrated in Figure 4. It would appear that virus-specific DNA is present equally in DNA from all fractions of nuclear chromatin, since euchromatin, heterochromatin, intermediate chromatin and total nuclear DNA did not differ significantly in their ability to accelerate the reassociation of MMTV DS-DNA. However, as these reactions were performed on an equal weight basis and the chromatin fractions are not present in equivalent proportions in the cell nucleus, it

might be argued that the similar acceleration occurring among chromatin fractions represents an enrichment of viral sequences in the heterochromatin and intermediate chromatin fractions. Alternatively, equal acceleration by all nuclear chromatin fractions could be explained by the possible heterogeneity of chromatin fractionated by the above procedure (Yasmineh and Yunis, 1970).

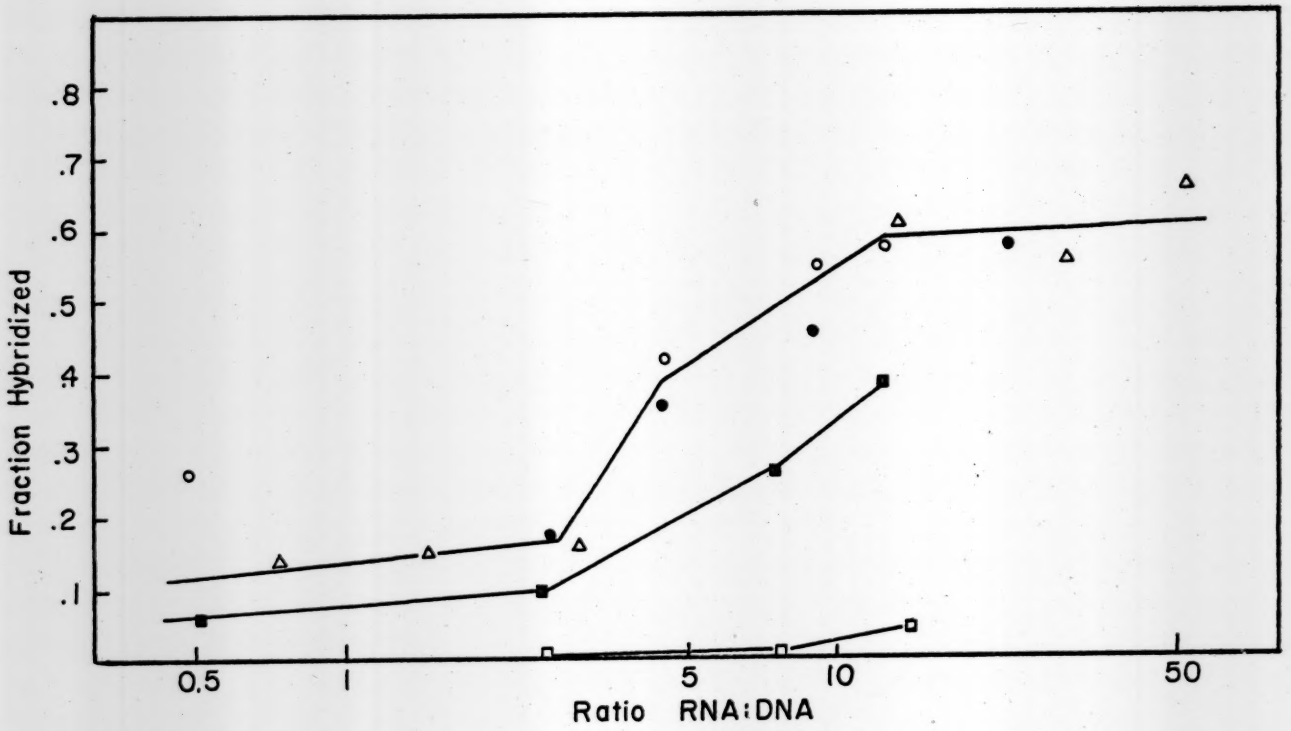
B. Characterization of MMTV cDNA:

Garapin et al. (1973) have reported that RSV single stranded DNA made in the presence of actinomycin D is not a uniform transcript of the viral RNA, but does contain virus-specific DNA which represents the entire 70S viral genome. Therefore, to obtain a more representative viral DNA, MMTV single stranded complementary DNA (cDNA) was synthesized in the presence of 100 µg/ml of actinomycin D according to the procedure outlined in the Materials and Methods.

1. The extent of MMTV 70S RNA transcribed into cDNA - The extent of transcription of MMTV 70S RNA into cDNA was examined by hybridizing increasing amounts of MMTV [^{32}P]cDNA with a constant amount of MMTV-S 70S [^{3}H]RNA, prepared from virus grown in BALB/c f C3H mammary tumor explants. Annealing of 70S RNA was determined by resistance to digestion with pancreatic ribonuclease (Garapin et al., 1973). The results of hybridization with four preparations of MMTV cDNA are shown in Figure 5. 62% of viral 70S RNA was protected from ribonuclease digestion after hybridization with a 15-50 fold excess of cDNA, indicating that MMTV RNA is not as extensively transcribed as RSV in

Figure 5. The hybridization of viral 70S RNA with MMTV cDNA.

MMTV-S [^{3}H]RNA (2.7 ng, 4.5X10^{5} cpm/µg) was annealed with variable amounts (1.33-138 ng) of four preparations of MMTV [^{32}P]cDNA (2.5X10^{5} cpm/µg; ○ , ● , △ , ▲) or various amounts (1.33-37 ng) of Moloney MuLV [^{32}P]cDNA (2.5X10^{5} cpm/µg; ■). MuLV 70S [^{3}H]RNA (2.9 ng, 4.5X10^{5} cpm/µg) was hybridized with increasing amounts (1.46-36.5 ng) of Moloney MuLV [^{32}P]cDNA (2.5X10^{5} cpm/µg). Hybridization was performed in 10 µl or 40 µl reaction volumes containing 46.4 ng of HeLa cell RNA, 0.4 M PB or 0.6 M PB, at 68°C for 60 to 70 hours. The fraction hybridized was determined by resistance to digestion with pancreatic ribonuclease (50 µg/ml, 37°C, for 30 min in 2XSSC) and was plotted against the DNA:RNA ratio on a semi-log scale. Corrections have been made for intrinsic ribonuclease resistance (4.5-14.7% MMTV 70S [^{3}H]RNA; 27%, MuLV 70S [^{3}H]RNA).



the presence of actinomycin D. Furthermore, the high DNA:RNA ratios necessary to obtain 60% ribonuclease resistance suggest a substantial amount of preferential transcription. 62% viral genome representation in MMTV cDNA is a minimum estimate as the MMTV obtained from RIII milk and the MMTV released by BALB/c f C3H cells may not have complete genome homology.

The MMTV-S 70S [^{3}H]RNA used in these experiments was prepared from virus released by BALB/c f C3H mammary tumor explants, and it was possible that such explants might be producing MuLV as well as MMTV (Hilgers et al., 1971; Yagi, 1973). Since MMTV and MuLV do not cross react in hybridization reactions (Varmus et al., 1973b), contamination with MuLV could explain why MMTV cDNA did not hybridize with greater than 62% of the viral RNA. This possibility was examined by hybridizing the putative MMTV 70S [^{3}H]RNA with increasing amounts of Moloney MuLV [^{32}P]cDNA. As a positive control similar hybridizations were performed using Gross MuLV 70S [^{3}H]RNA and Moloney-MuLV cDNA. At a DNA:RNA ratio of 12.5:1, MuLV cDNA annealed to 38% of the MuLV RNA, while at the same ratio it hybridized to less than 5% of the 70S RNA from virus producing mammary tumor cultures (Figure 5). Thus, BALB/c f C3H mammary tumor explants were not producing significant quantities of MuLV and the extent of viral transcription into MMTV cDNA does not appear to exceed approximately 60%.

2. Specificity of MMTV cDNA - MMTV [^{3}H]cDNA was not tested directly for its specificity to anneal with MMTV sequences by

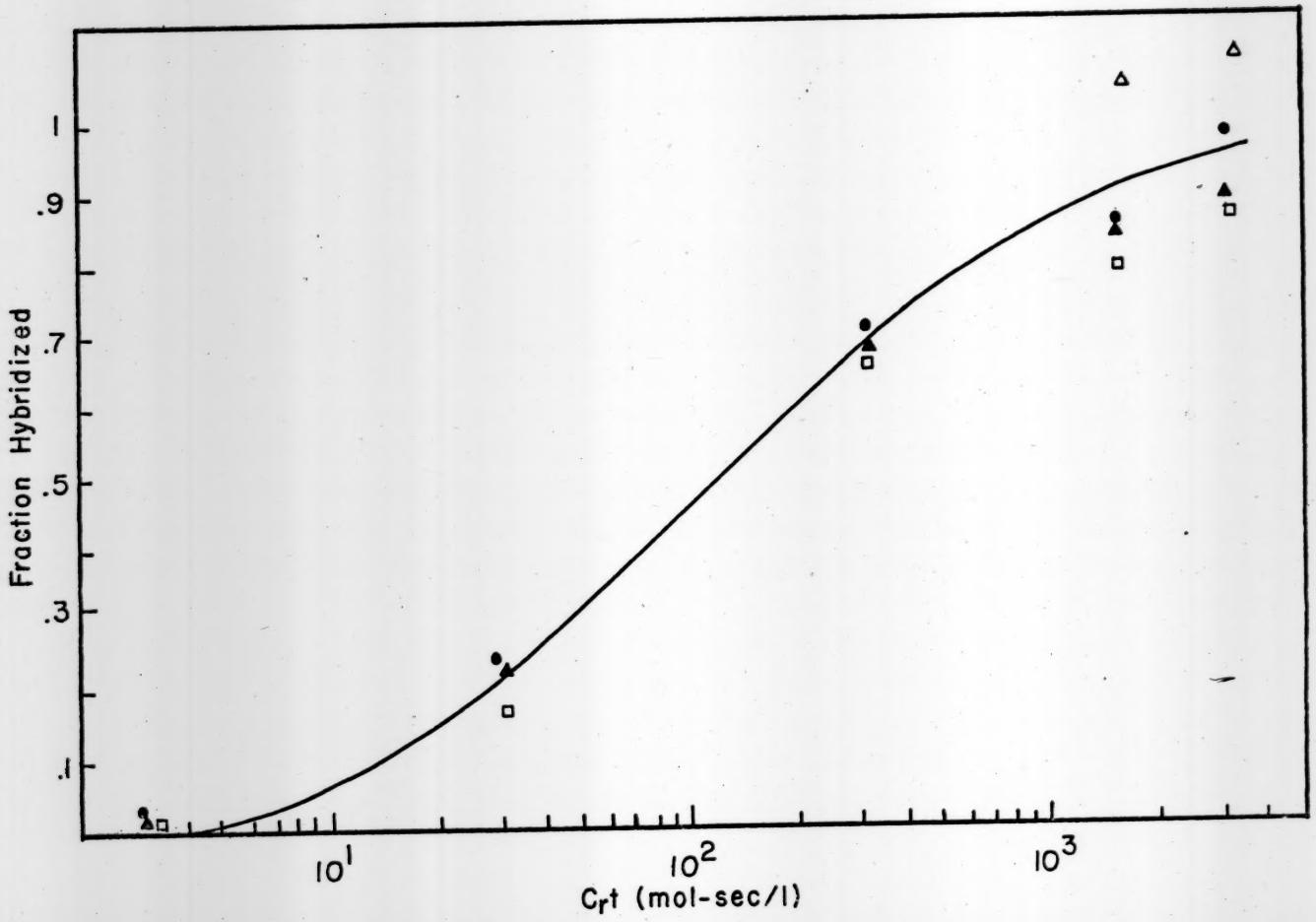
hybridizing it with MMTV or MuLV 70S RNA. However, when each preparation of cDNA was annealed with RNA from MMTV-producing cells, cDNA hybridized with approximately the same Crt1/2 ($1.3X10^2$, Figure 6) as MMTV cDNA which had reacted specifically with MMTV 70S RNA (Varmus et al., 1973b). This suggests that my preparations of MMTV cDNA were not significantly contaminated with MuLV transcripts and that they could hybridize specifically with MMTV sequences.

3. Maximum extent of hybridization obtainable with MMTV cDNA - To assess the extent to which MMTV cDNA was able to hybridize with viral nucleic acid, samples of four viral cDNA preparations were annealed with RNA from virus-producing C3H mammary tumors and assayed for resistance to digestion by S1 nuclease. At a Crt value of $3X10^3$ mol-sec/l, viral cDNA was hybridized 90-100% (Figure 6), indicating that at least 90% of the MMTV cDNA is capable of hybridization when enough complementary sequences are available.

4. Nucleotide composition - The nucleotide composition of MMTV cDNA was determined in collaboration with Dr. J. Taylor. Viral DNA was digested with a mixture of micrococcal nuclease and splenic phosphodiesterase and the nucleotides were separated by paper electrophoresis (Taylor et al., 1972). Results are given in Table IV along with the nucleotide composition for MMTV RNA determined by Lyons and Moore (1965). There appears to be a lower proportion of deoxycytidine in the cDNA than one would expect from the guanosine content of MMTV RNA. This could mean that sequences

Figure 6. The hybridization of MMTV cDNA to RNA from MMTV producing tissue.

Four preparations of MMT viral [^{3}H]cDNA (0.036 - .058 ng, 1.6 - 3 X 10^7 cpm/µg) were hybridized to increasing quantities of RNA from a C3H mouse mammary tumor (0.14 ng - 0.14 mg). Hybridizations were performed in 0.3 M NaCl, 0.025 M Tris:HCl, pH 7.4, 0.001 M EDTA, for 40 hours at 68°C in 50 µl reaction volumes. The fraction of MMTV cDNA hybridized was determined by digestion with S1 nuclease and plotted against the Crt in mol-sec/l on a semi-log scale. Data is corrected for intrinsic S1 resistance (3.2-4.5%). Preparation 1 (●), 2 (△), 3 (▲), 4 (□).



rich in guanosine are not being transcribed into DNA or are being transcribed less frequently than other sequences. Since the "relative percentage" in Table IV is based on the specific activity given by the supplier, a possible error in the actual specific activity for dCTP might also account for the low percentage determined for deoxycytidine in MMTV cDNA. The percentage of thymidine was not unusually high, suggesting that MMTV cDNA does not contain stretches of poly (T). Such regions could interfere with the specificity of viral cDNA when hybridizing to cellular RNA by annealing to adenosine rich areas common to many messenger ribonucleic acids. Varmus et al. (1973b) have also shown that MMTV cDNA does not contain poly (T) by its failure to hybridize with poly (A).

TABLE IV

The nucleotide composition of MMTV cDNA and MMTV RNA

MMTV cDNA					MMTV RNA*	
	cpm	specific activity Ci/mM	cpm / S.A.	relative percentage		relative percentage
dC	298	30	9.9	15.5	G	30
dA	360	16.8	21.4	33.3	U	29
dG	166	9.8	17.0	26.7	C	22
T	210	13.4	15.7	24.5	A	19

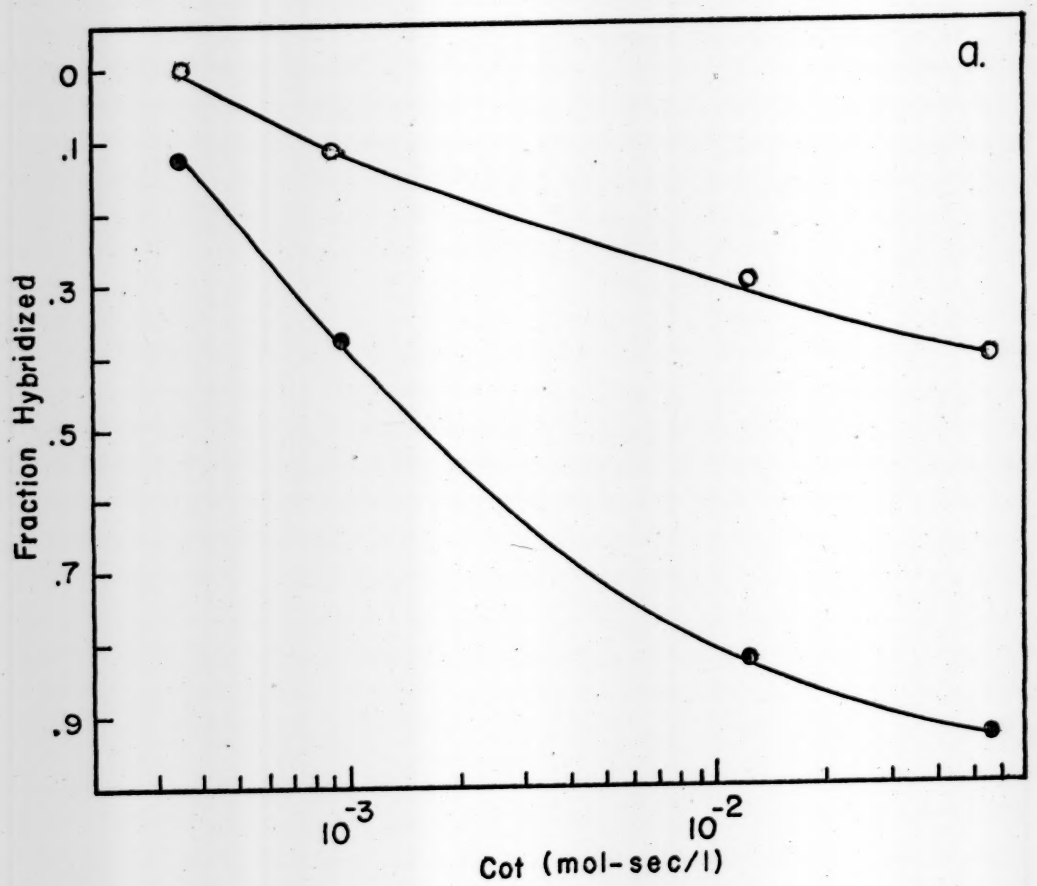
* Taken from Lyons and Moore (1965).

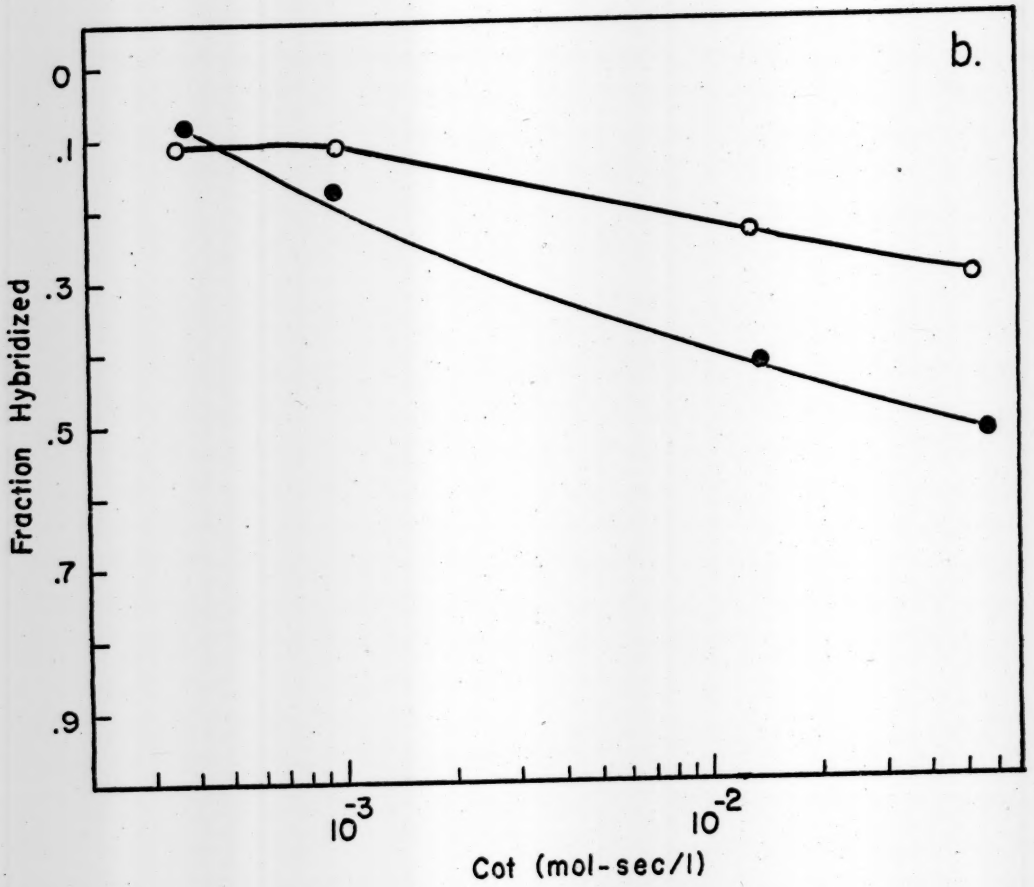
5. Homology between MMTV DS-DNA and MMTV cDNA - To determine what percentage of viral sequences were shared by MMTV double and single stranded DNA, MMTV [^{3}H]cDNA was hybridized to MMTV [^{32}P]DS-DNA at a DS:cDNA ratio of 62:1. With this ratio, DS-DNA should have been in sufficient excess to allow hybridization of cDNA without significantly affecting the reassociation kinetics of DS-DNA (Bishop, 1972). Reaction conditions have been described in Materials and Methods. The fraction of hybridization and reassociation was determined by hydroxylapatite fractionation and by digestion with S1 nuclease. When assayed by fractionation of HAP, 94% of the MMTV DS-DNA reannealed while only 42% of the MMTV cDNA was hybridized (Figure 7a). At the same Cot value ($5.5X10^{2}$ mol-sec/l), MMTV DS-DNA was 52% resistant and MMTV cDNA 30% resistant to digestion by S1 nuclease (Figure 7b). It would appear from these data that approximately 30% of the cDNA contained sequences homologous with DS-DNA, and that sequences represented in the non-annealing cDNA were either not present or present in small amounts in DS-DNA. This suggests that MMTV cDNA could possibly detect MMTV specific sequences not detectable with DS-DNA.

The large difference in the final extent of reassocation for DS-DNA (42%) when assayed by two different techniques was much greater than expected on the basis of variation between the HAP and S1 assay methods (Varmus et al., 1974b) and can be explained by the presence of minus stranded "tails" on DS-DNA molecules (Deng; Quintrell, unpublished observation). Since cDNA and DS-DNA "tails"

Figure 7. The hybridization of MMTV cDNA with MMTV virus DS-DNA.

MMTV [^{32}P]DS-DNA (1.7 X 10^5 cpm/µg, ●) was reassociated in the presence of MMT virus [^{3}H]cDNA (3 X 10^7 cpm/µg, o) at a DS:SS ratio of 62:1. DNA was denatured for three minutes at 100°C and hybridized in 0.6 M NaCl, at 68°C, to a maximum Cot value of 5.5 X 10^{-2} mol-sec/l. Annealing was assayed by resistance to S1 nuclease (panel a) or hydroxylapatite fractionation (panel b) and plotted against Cot (mol-sec/l) on a semi-log scale.





have the same polarity of nucleotide sequence, it is possible that DS-DNA may contain sequences homologous with cDNA which would not have been detected by these experiments.

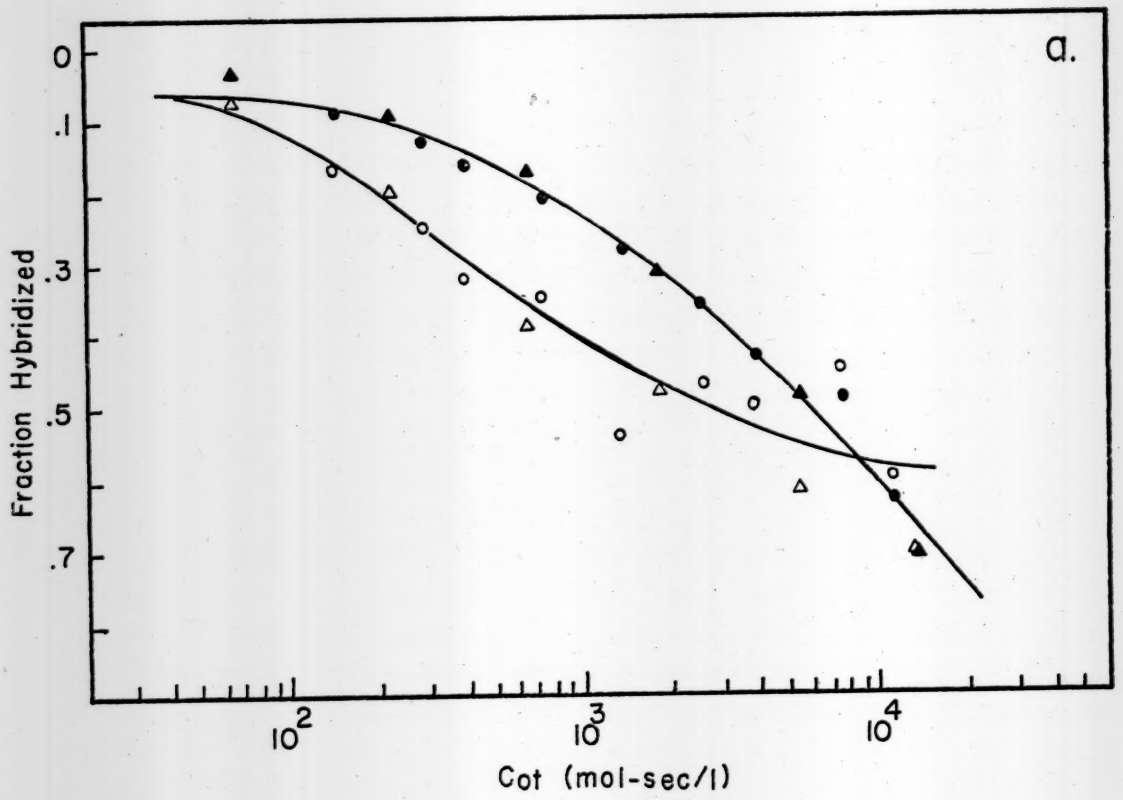
C. Quantitation of Mouse Mammary Tumor Viral Genes in Mouse Cell DNA by Hybridization with MMTV cDNA:

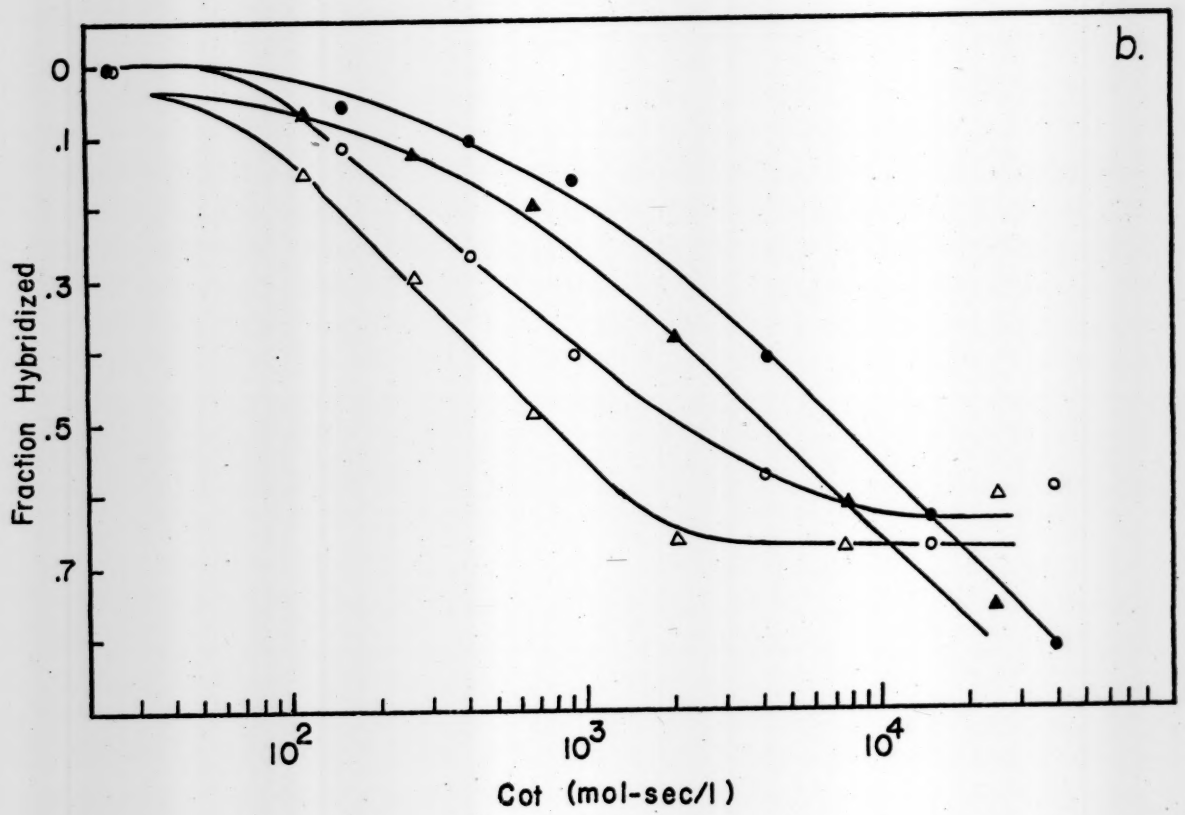
Because of the greater representation of MMTV nucleotide sequences in MMTV cDNA than in MMTV DS-DNA, the number of MMT viral genes present in the DNA of mice with high and low incidences of mammary tumors was re-examined by following the reassociation of unlabeled cellular DNA with small amounts of MMTV [^{3}H]cDNA. Hybridizations were performed with six strains of mice, as described in Materials and Methods, at a mouse DNA:virus DNA mass ratio near $1X10^7$. Preliminary experiments showed that maximum hybridization of MMTV cDNA was achieved at this ratio. Furthermore, at a ratio of 10^7 there should be a sufficient excess of unlabeled viral sequences to enable the reassociation to proceed without being significantly affected by the hybridization reaction (see Discussion). The reassociation of mouse sequences, presumably represented only once per haploid genome, was followed within the same reaction by including trace amounts of mouse "unique sequence" [C^{14}]DNA.

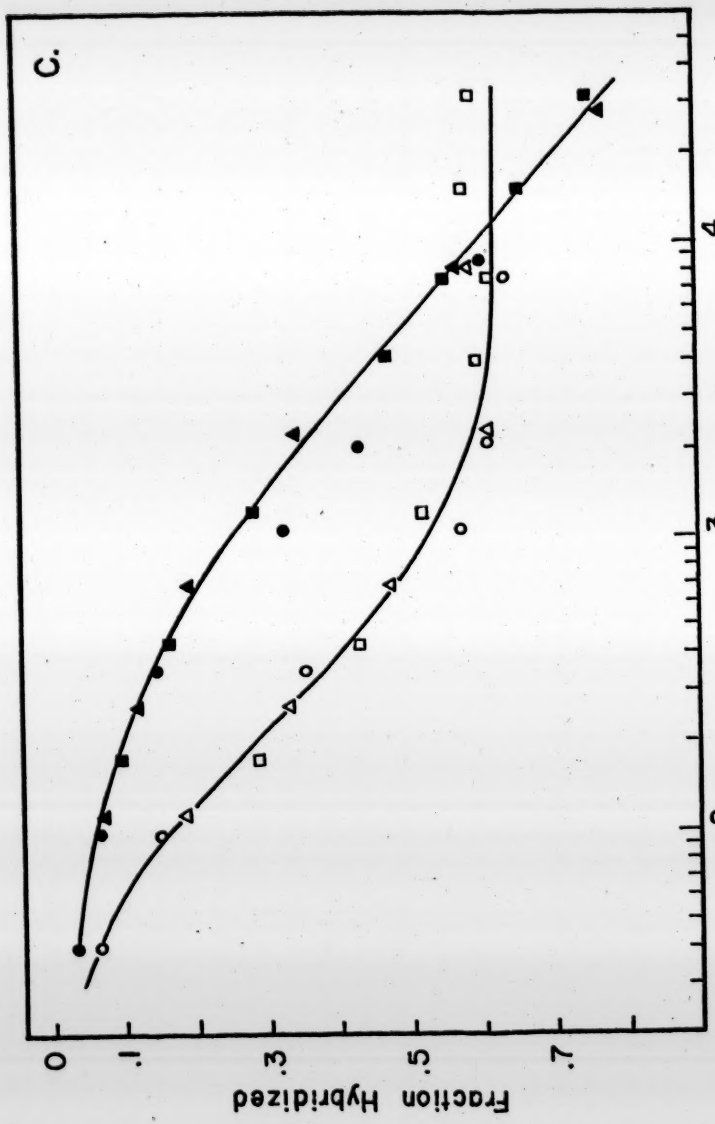
Results for strains RIII, GR, C3H, C3HeB/Fe, C57Bl/6 and BALB/c are illustrated in Figure 8a, b, c, d, e and f. The fraction hybridized (as determined by S1 nuclear digestion) is plotted against the Cot on a semi-log scale (according to the convention of

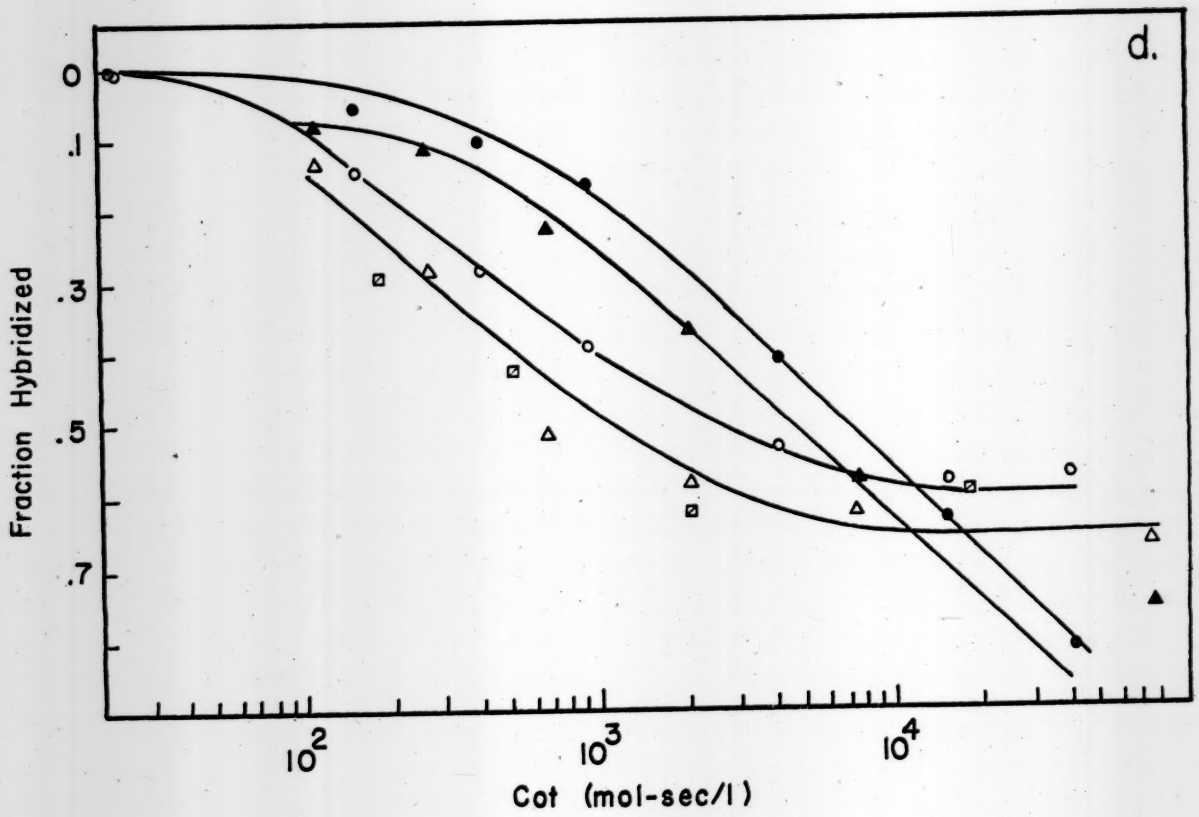
Figure 8. The hybridization of MMTV cDNA and mouse "unique sequence" DNA to mouse cell DNA.

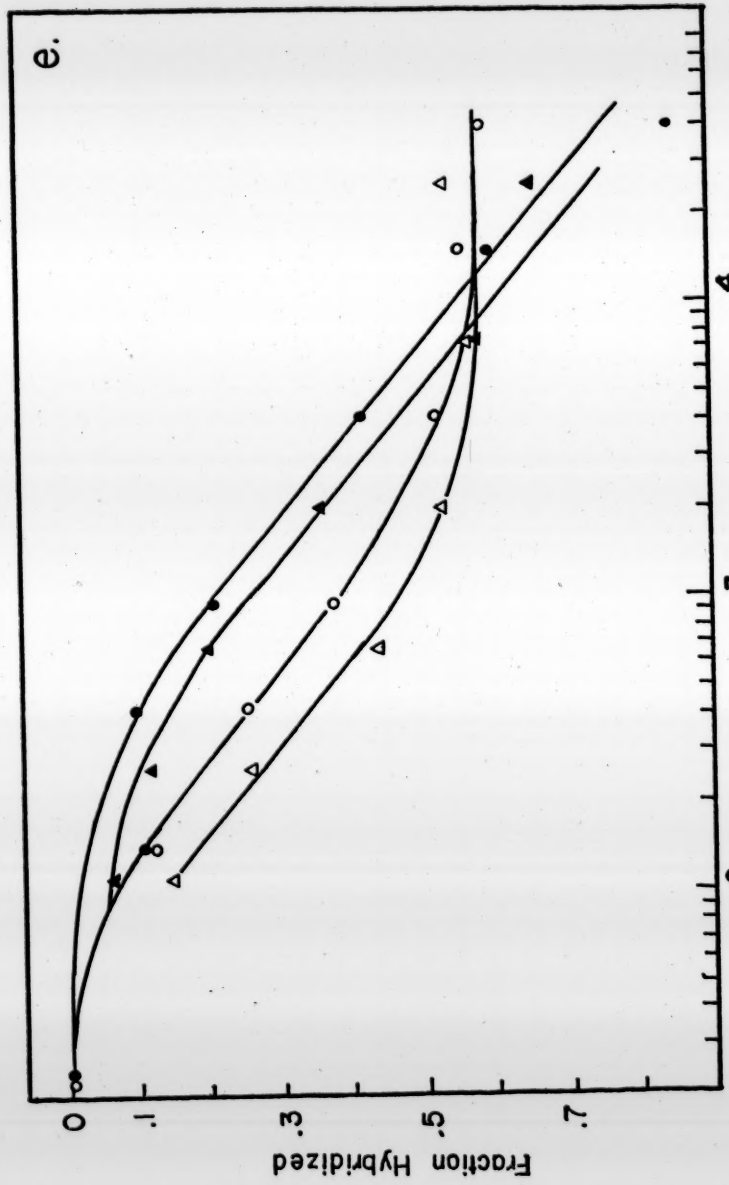
MMTV [^{3}H]cDNA (0.22-0.51 ng/ml, 1.6-3 X 10^{7} cpm/µg) and mouse "unique sequence" [c^{14}]DNA (257 ng/ml, 1.45 X 10^{4} cpm/µg) were hybridized to 4 mg/ml of unlabeled mouse DNA in 0.6 M NaCl, 68°C. Samples (0.25 ml) were removed periodically to S1 buffer, assayed for resistance to digestion by S1 nuclease and the fraction hybridized was plotted against Cot (mol-sec/l) for unlabeled mouse DNA on a semi-log scale. Hybridization of MMTV cDNA with (a) RIII, (b) C3H, (c) GR, (d) C3HeB/Fe, (e) C57BL/6 and (f) BALB/c DNA is illustrated by open symbols, (o , Δ , □). Closed symbols (● , ▲ , ■), represent hybridization of "unique sequence" DNA. In a separate experiment, MMTV [^{3}H]cDNA was annealed with C3HeB/Fe cell DNA under the conditions outlined above. Hybridized DNA was isolated by fractionation on HAP, dialyzed, concentrated, denatured and reassociated. Samples were assayed for resistance to digestion by S1 nuclease and the fraction hybridized was plotted against the Cot (mol-sec/l) for C3HeB/Fe cell DNA (d, ⊠).






C.
Fraction Hybridized
0
.1
.3
.5
.7
10^2
10^3
10^4
Cot (mol-sec/l)




e.
Fraction Hybridized
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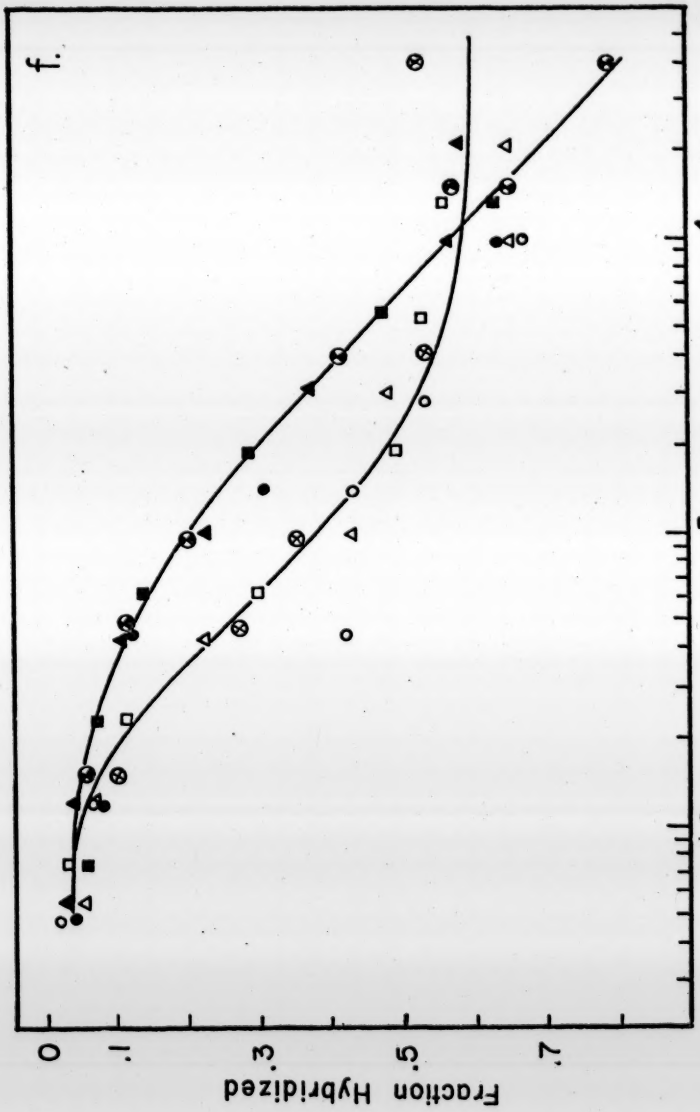
.5
.7
10^2
10^3
10^4
Cot (mol-sec/l)


f.
Fraction Hybridized
0
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10^2
10^3
10^4
Cot (mol-sec/l)

Britten and Kohne, 1968). Figures for the hybridization with RIII, GR and BALB/c DNA are composite drawings using data from experiments performed with different preparations of viral and mouse cell DNA. "Unique sequence" DNA hybridized similarly with each mouse strain, reaching a maximum of 80% annealing at a Cot of $4X10^4$ mol-sec/l without beginning to plateau. MMTV cDNA hybridized at a faster rate than "unique sequence" DNA, reaching a plateau when only 59-71% of the cDNA had been annealed. The final extent of hybridization varied within the same species and from species to species. This early termination of hybridization appears to be inherent to reactions involving cDNA synthesized with RNA tumor virus RNA directed DNA polymerase (see Discussion).

To determine if unannealed MMTV cDNA could hybridize further with additional cell DNA, cDNA (0.12 ng) was hybridized with C3HeB/Fe cell DNA (2 mg) at 4 mg/ml of cell DNA in 0.6 M Na^+, at 68°C, to a Cot value of $2.7X10^4$ mol-sec/l. A sample was removed and the percentage of hybridized cDNA was determined by resistance to digestion with S1 nuclease. Two milligrams of freshly denatured C3HeB/Fe DNA were then added to the remaining reaction mixture, the sodium ion concentration adjusted to 0.6M, and the entire sample was reincubated to a Cot of $2X10^4$ mol-sec/l. A sample was removed, assayed as before, and the hybridization was repeated with the addition of 2 mg of freshly denatured C3HeB/Fe cell DNA. The final extent of hybridization was assayed by S1 resistance at a Cot of $2.7X10^4$ mol-sec/l. Fifty-five percent of the MMTV cDNA was annealed

in the first reaction, 67% in the second and 74% in the last. Since the extent of hybridization could be improved by the sequential addition of DNA, it appears that there were insufficient homologous viral sequences in the starting amount of cell DNA. The gradual increase in additional hybridization of cDNA with large amounts of cell DNA indicates that DNA sequences remaining unhybridized are probably very redundant and are represented few times in the cell genome.

To improve the analysis of viral sequences in cell DNA, an attempt was made to remove possible redundant sequences from cDNA. MMTV cDNA (0.22 ng) was reacted with C3HeB/Fe cell DNA (4 mg) at 4 mg/ml of DNA in 0.6 M Na^+ at 68°C to a Cot of $3X10^4$ mol-sec/l. Hybridized DNA was then separated from unannealed DNA by fractionation on a hydroxylapatite column. Seventy percent (by HAP) of the DNA was recovered as double stranded. This DNA was dialyzed, concentrated, denatured and reassociated as before. It was expected that previously hybridized cDNA would not contain a large population of redundant sequences and would therefore reanneal completely. Instead, it hybridized with the same kinetics and to the same extent (60%) as complete cDNA (Figure 8d). Similar results have been found for the reannealing of MuLV cDNA which had been hybridized and isolated on HAP (Lowy, personal communication). These findings indicate that incomplete hybridization of cDNA may be due to problems involving the kinetics of interaction between cDNA and homologous viral sequences in cellular DNA.

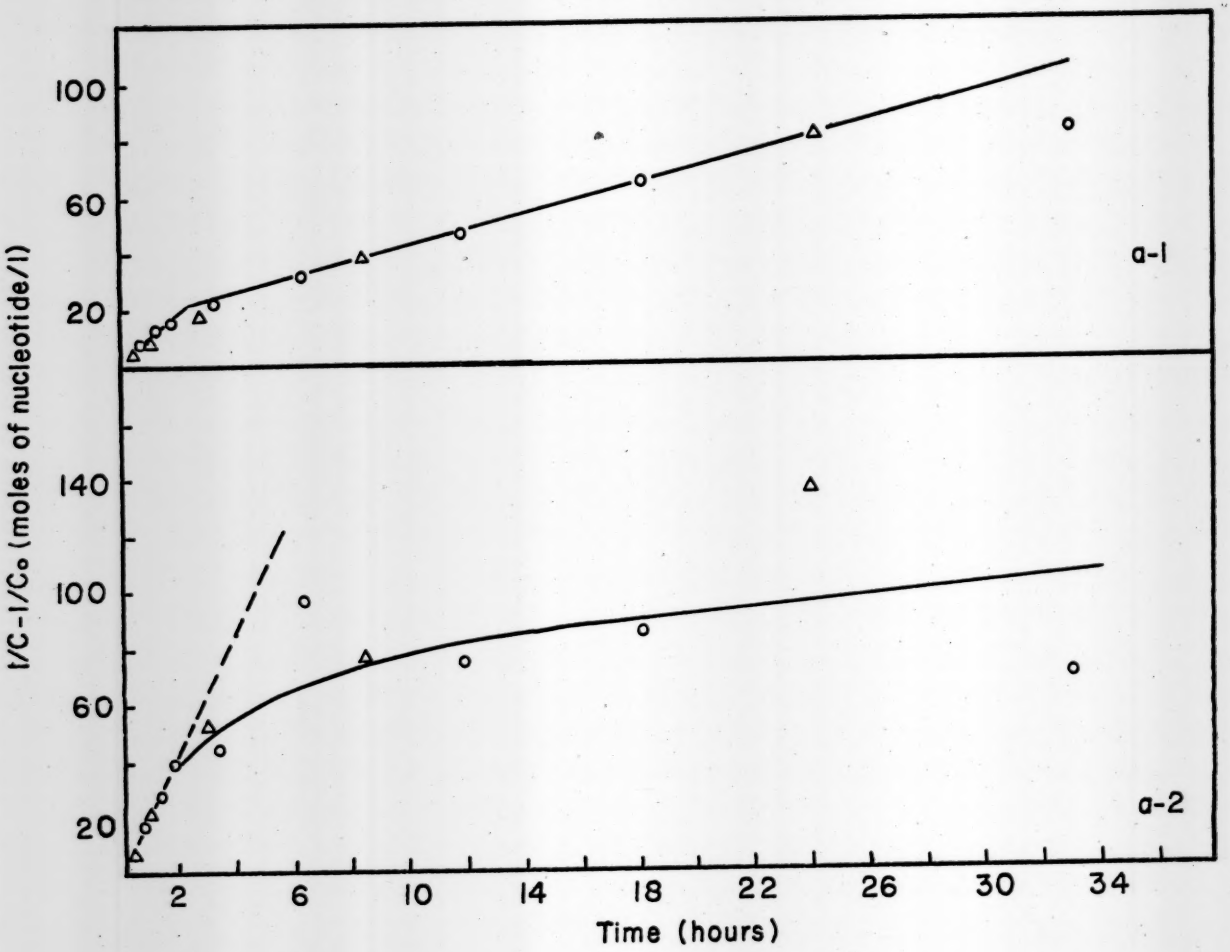
The number of viral genes in cell DNA can be estimated by comparing the Cot1/2 for the hybridization of "unique sequence" DNA with that for MMTV cDNA. If one assumes that the reassociation of mouse mammary tumor virus-specific sequences is accurately reflected by the curves in Figures 8a-f, the Cot1/2 must be taken as the Cot value half way between the minimum and maximum points of hybridization. The evaluation of data becomes difficult when there is variation in the final points of the reaction as in Figures 8a, c and f and on a log scale where small changes in the curve are reflected as large changes in copy number. Therefore, values determined from these curves are subject to errors introduced by the subjective decision concerning the positioning of curves and in choosing the minimum and maximum points of hybridization.

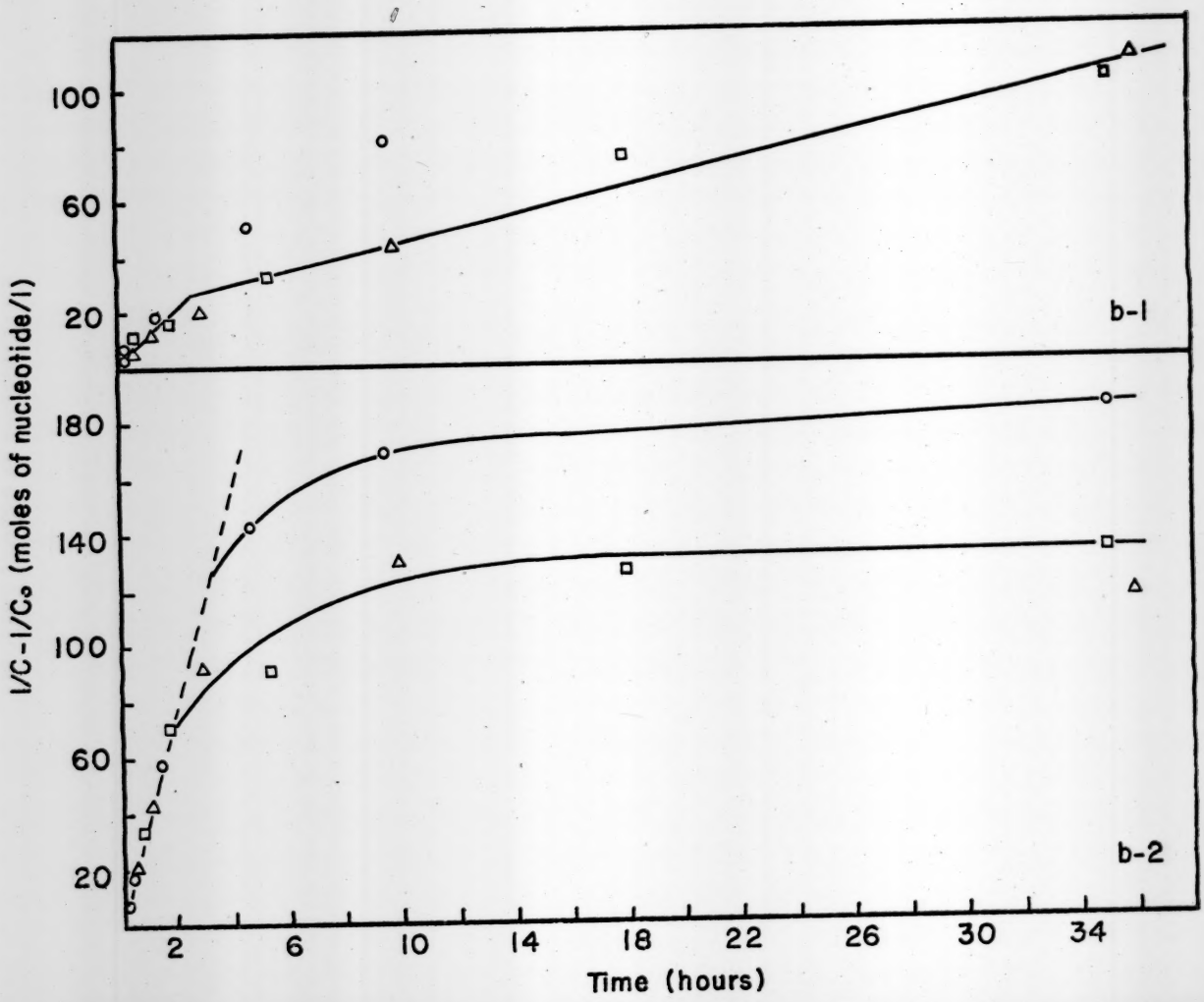
To avoid these errors, data was re-expressed according to the method of Wetmur and Davidson (1968) as $1/C - 1/C_0$ versus time; C is the concentration of single stranded DNA (moles of nucleotide/l), and C_0 is the original total concentration of DNA (moles of nucleotide/l) at the start of the reaction. When expressed in this way, reassociation of sequences with similar complexity appears as a straight line and the slope of the line is equal to the second order rate constant for the reaction.

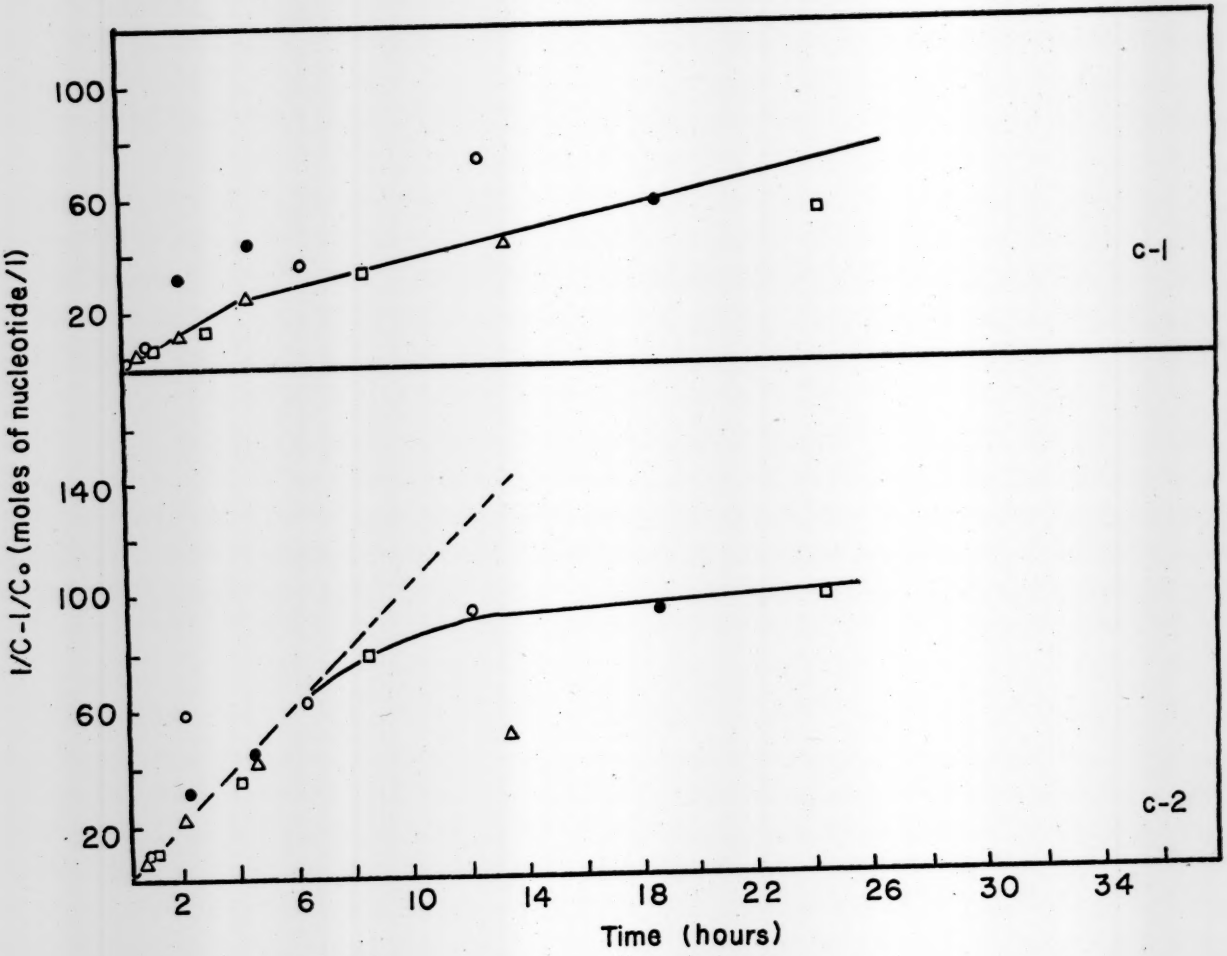
Similar results were obtained for all strains of mice when hybridization data was replotted; examples for strains RIII, GR and BALB/c are presented in Figure 9a, b and c. In each case the hybridization of "unique sequence" DNA appeared biphasic, suggesting

Figure 9. The hybridization of MMTV cDNA and mouse "unique sequence" DNA to mouse cell DNA.

MMTV [^{3}H]cDNA (0.22-0.51 ng/ml, 1.6-3 X 10^{7} cpm/µg) and mouse "unique sequence" [c^{14}] DNA (257 mg/ml, 1.45 X 10^{4} cpm/µg) were hybridized to 4 mg/ml of mouse cell DNA in 0.6 M NaCl, 68°C. Samples (0.25 ml) were removed periodically to S1 buffer and assayed for resistance to digestion by S1 nuclease. The results are expressed as $1/C-1/C_o$ (moles of nucleotide) versus time (hours). Where C = concentration of single stranded DNA and C_o the original total concentration of DNA. The upper half of each panel (1) represents hybridization of [c^{14}] "unique sequence" DNA; the lower half (2) represents hybridization of [^{3}H]cDNA. Hybridization with RIII cell DNA (a), GR cell DNA (b), BALB/c cell DNA (c).


1/C-1/Co (moles of nucleotide/l)
100
60
20
a-1
140
100
60
20
a-2
2
6
10
14
18
22
26
30
34
Time (hours)


1/C-1/C₀ (moles of nucleotide/l)
100
60
20
b-1
180
140
100
60
20
b-2
2
6
10
14
18
22
26
30
34
Time (hours)


100
60
20
c-1
140
100
60
20
c-2
2
6
10
14
18
22
26
30
34
Time (hours)
1/C-1/Co (moles of nucleotide/l)

that "unique sequence" DNA was actually composed of more than one family of sequences. This finding should have been expected as the Cot value of $2.5X10^2$ mol-sec/l used for the preparation of "unique sequence" DNA occurs in the area of transition between reiterated and unique sequences in the reassociation of complete mouse cell DNA (McConaughy et al., 1969). And, contamination of "unique sequence" DNA by redundant sequences has been reported for mouse cell "unique sequence" DNA purified by the reassociation of the mouse cell DNA to a Cot of $2X10^2$ mol-sec/l followed by a single passage over hydroxylapatite (Grouse et al., 1972). Therefore the early portion of the curves (points less than 2 hr, Cot less than 10^3 mol-sec/l) was considered to represent reassociation of redundant sequences, while the later portion (points greater than 2 hr or 10^3 mol-sec/l) was considered representative of truly unique sequences, and its slope was used in the determination of viral copy numbers. The hybridization of MMTV cDNA appeared more as a curve than a straight line. This signified a great deal of heterogeneity in reaction rates, and might have been anticipated given the unequal representation of viral sequences in the MMTV cDNA. A straight line could be drawn through the early points in each reaction and the slope of this line was used in calculating the number of viral copies. These early points most likely represent a part of the reaction where there was an excess of virus-specific sequences. A viral copy number was computed for each strain of mouse by dividing the rate constant for hybridization by the rate constant for "unique sequence" DNA. The rate constants

and copy numbers for each reaction are listed in Table V.

Significant differences in copy numbers were obtained among some of the mouse strains. Strains RIII, C3H, C3HeB/Fe and C57BL/6 were quite similar, containing 13-17 viral copies/diploid cell. BALB/c mice were somewhat lower with 9 copies, and strain GR was 2-3.5 times higher than the other strains of mice with 32 copies. The viral copy numbers were reproducible using different preparations of MMTV cDNA with cell DNA prepared at different times from the same mouse strain (Figure 9a, b and c). With RIII, C3H, C3HeB/Fe and C57BL/6 mouse strains no direct correlation can be drawn between the number of viral copies for a particular species and its incidence of mammary tumors. The lower number of copies in BALB/c and the higher number in GR mice do correspond with their incidence of mammary tumors; however, the significance of this correlation is unclear.

D. The Fidelity of Base Pairing Between MMTV cDNA and Mouse DNA:

The fidelity of base pairing in hybrids formed between MMTV cDNA and mouse DNA was determined by assaying their thermal stability. Hybrids were denatured by thermal elution chromatography from hydroxylapatite (Miyazawa and Thomas, 1965) or heating samples to various temperatures in 0.02M Tris:HCl, pH 7.4, chilling immediately in an ice water bath and assaying for the extent of denaturation by digestion with S1 nuclease (Varmus et al., 1973b).

TABLE V

The number of MMTV genomes per diploid mouse cell determined by hybridization of MMTV cDNA

Mouse strain	Rate constant (1/mol-sec) "unique sequence"	Rate constant (1/mol-sec) MMTV cDNA	haploid copy number	average copy number	diploid copy number
RIII*	$7.3X10^{-4}$	$5.5X10^{-3}$	7.5	7.5	15
C3H	$8.5X10^{-4}$	$4.6X10^{-3}$	5.4	6.3	13
	$9.6X10^{-4}$	$6.9X10^{-3}$	7.2		
C3HeB/ Fe	$8.4X10^{-4}$	$5.1X10^{-3}$	6.1	8	16
	$8.1X10^{-4}$	$7.9X10^{-3}$	9.8		
GR*	$6.8X10^{-4}$	$1.1X10^{-2}$	16.2	16.2	32
C57BL/6	$4.7X10^{-4}$	$4.6X10^{-3}$	9.8	8.5	17
	$7.7X10^{-4}$	$5.6X10^{-3}$	7.3		
BALB/c*	$6.3X10$	$2.8X10^{-3}$	4.5	4.5	9

* Data represents at least three separate experiments.

1. Thermal elution chromatography - When assayed by thermal elution chromatography, there were no detectable differences in the denaturation of hybrids formed between cDNA and DNA from each of the mouse strains. Only one denaturation curve was drawn for all hybrids with a Tmi[1] of 75.5°C (Figure 10). To establish a control for any aberrant fluctuations in elution between HAP columns, a standard of mouse "unique sequence" DNA was included within each experiment. Figure 10b shows that "unique sequence" DNA was uniformly eluted from all HAP columns with Tmi of 78.5°C, indicating that no fluctuations had occurred among the columns. A Tmi of 78.5°C was surprisingly low, however, since McConaughy and McCarthy (1970), using the same procedure, had reported a Tm[2] of 89°C for native and 86°C for renatured mouse DNA. An explanation for this difference is that the binding capacity of the HAP used in the present experiments had been altered by boiling for 10-15 minutes in 0.12 M PB before preparing the columns. The HAP was boiled to remove trapped air and to improve the eluting flow rate of the columns. Recent experiments by Martinson (1973) have shown that when boiled HAP is used in thermal elution experiments, it has a progressive decrease in affinity for adsorbed DNA which is accompanied by a reduction in the molarity of elution (Me) for double stranded DNA. During chromatography, if the Me should

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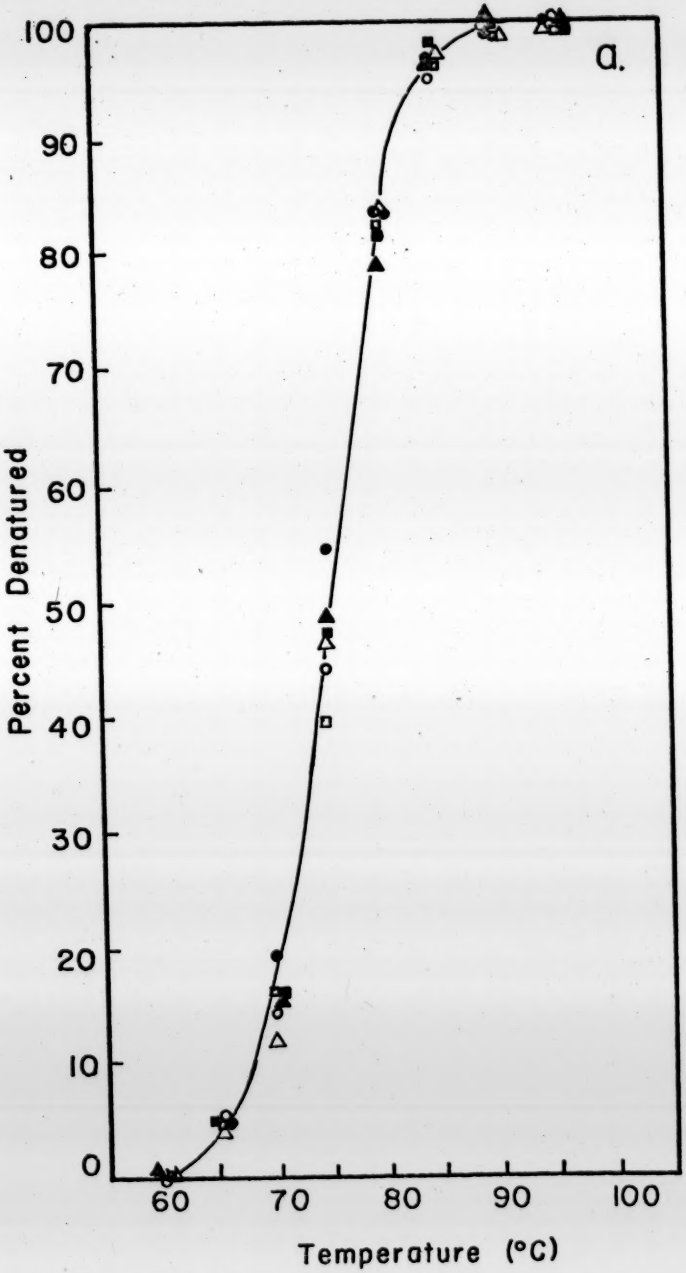
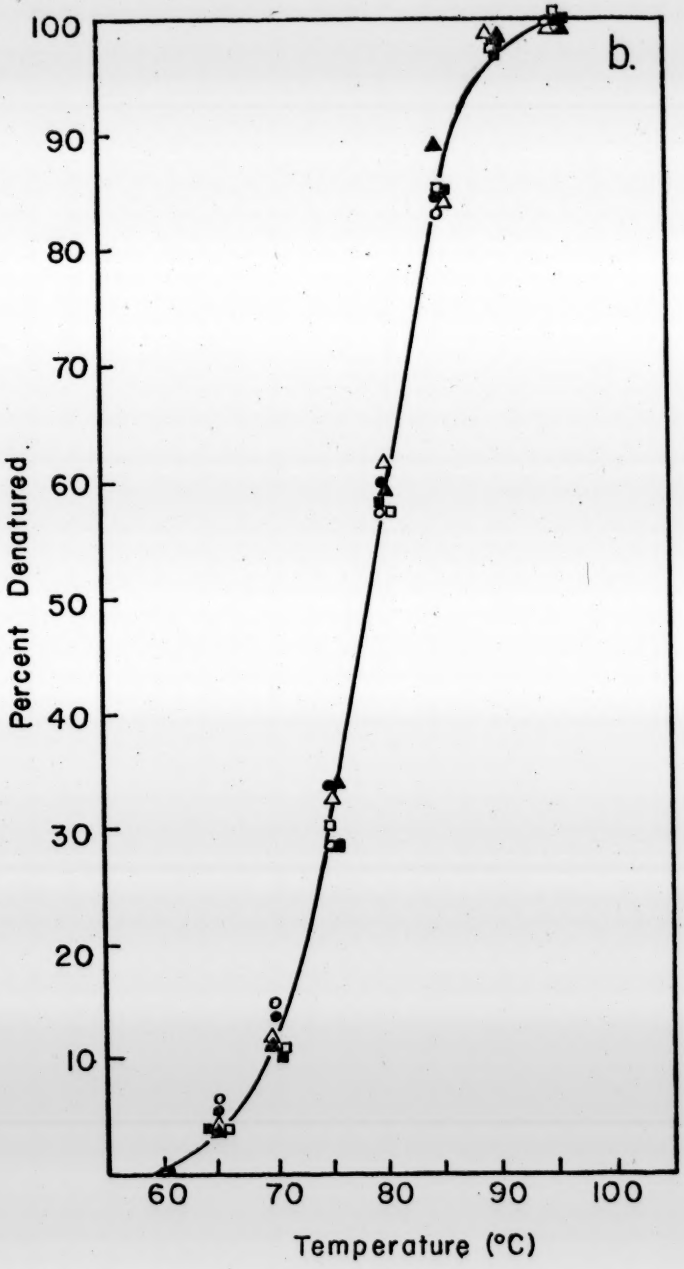
[1] Tmi = temperature by which 50% "irreversible" strand separation has occurred (Crothers et al., 1965).

[2] Tm = mean temperature of denaturation.

Figure 10. The thermal stability of hybrids formed between MMTV cDNA and mouse cellular DNA, assayed by thermal elution from hydroxylapatite columns.

Hybridizations were carried out as described in Figure 11, except that 0.4 M PB was substituted for 0.6 M NaCl and "unique sequence" [C^{14}]DNA ($1.3X10^4$ cpm/µg) was included in each reaction. Reactions were diluted to 0.12 M phosphate buffer and hybridized DNA was adsorbed to hydroxylapatite columns at 60°C. The temperature of the columns was raised by increments of 5°C and denatured DNA removed by elution with 0.12 M PB. The cumulative percent denatured DNA was plotted against temperature. Thermal denaturation of MMTV cDNA hybridized to RIII (o), C3H (●), GR (△), C3HeB/Fe (▲), C57BL/6 (□), and BALB/c (■), DNA (a). Thermal denaturation of [C^{14}] "unique sequence" DNA (b).


a.
Percent Denatured
100
90
80
70
60
50
40
30
20
10
0
60
70
80
90
100
Temperature (°C)


b.
Percent Denatured
100
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70
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30
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10
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80
90
100
Temperature (°C)

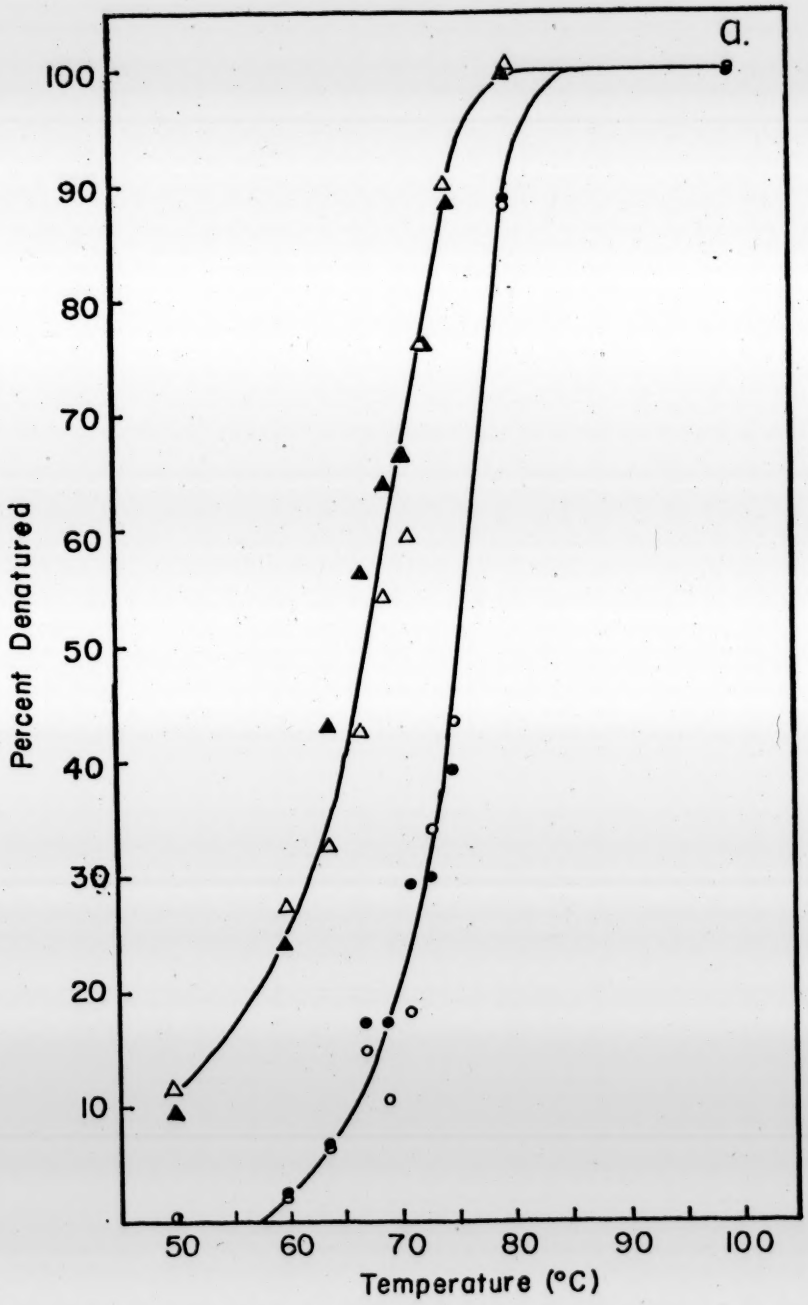
fall below the molarity of the eluting buffer before DNA is denatured, native DNA will be eluted along with single stranded DNA, causing an apparent decrease in the Tmi. This might also explain why no differences were observed in the denaturation profiles for various hybrids since elution of native DNA could mask small differences in the actual Tmi.

2. Thermal denaturation assayed by S1 nuclease digestion - When denaturation was assayed by digestion with S1 nuclease, differences in denaturation profiles were resolved between hybrids formed with RIII DNA and those formed with DNA from other strains of mice (Figure 11a,b,c, Table VI). Denaturation curves for C3H, C3HeB/Fe and BALB/c hybrids were similar, having a Tm approximately 4°C lower than the Tm for RIII hybrids. GR and C57BL/6 showed the greatest differences in Tm with decreases of 8°C and 6.5°C respectively.

MMTV DS-DNA was also denatured and assayed for resistance to S1 nuclease digestion on the assumption that it would represent perfectly base-paired MMTV DNA hybrids. Although it denatured with a slightly higher Tm than most of the MMTV cDNA hybrids, it had a Tm 3°C lower than hybrids formed with RIII DNA. One possible explanation for this difference is that only 35-50% of the DS-DNA molecule is actually involved in duplex formation, while the remaining portion is single stranded (see Results, B-5). Therefore the double stranded portion of MMTV DS-DNA is probably much smaller than the double stranded segment in hybrids formed between MMTV cDNA and RIII DNA. This difference in size could

Figure 11. The thermal denaturation of hybrids formed between MMTV cDNA and mouse cellular DNA, assayed by S1 nuclease digestion.

MMT virus [^{3}H]cDNA (0.153 ng/ml-0.25 ng, 2.5-3 X 10^7 cpm/µg) was hybridized with 4 mg/ml of cellular DNA in 0.6 M NaCl at 68°C, to a Cot value near 10^4 mol-s/l. The entire reaction was ethanol precipitated and resuspended in 0.02 M Tris:HCl, pH 7.4. Samples from each hybridization were heated at the desired temperature for fifteen minutes and immediately chilled in an ice water bath. Each sample was assayed by digestion with S1 nuclease for the precentage of denatured hybrid and plotted against the temperature. All figures represent duplicate experiments and were corrected for unhybridized MMT virus cDNA present before denaturation (30-40%). MMTV [^{3}H]DS-DNA was heated and assayed as above. (a) Thermal denaturation of MMT virus DS-DNA (o), and MMT virus cDNA annealed with GR (Δ) or RIII (●) DNA. (b) Thermal denaturation of MMT virus cDNA annealed with C3H (o) or C3HeB/Fe (●) DNA. (c) Thermal denaturation of MMT virus cDNA annealed with BALB/c (o) or C57BL/6 (●) DNA. The broken line in panels b and c represents the denaturation of hybrids formed with MMTV cDNA and RIII cell DNA (panel a).


a.
100
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10
Percent Denatured
50
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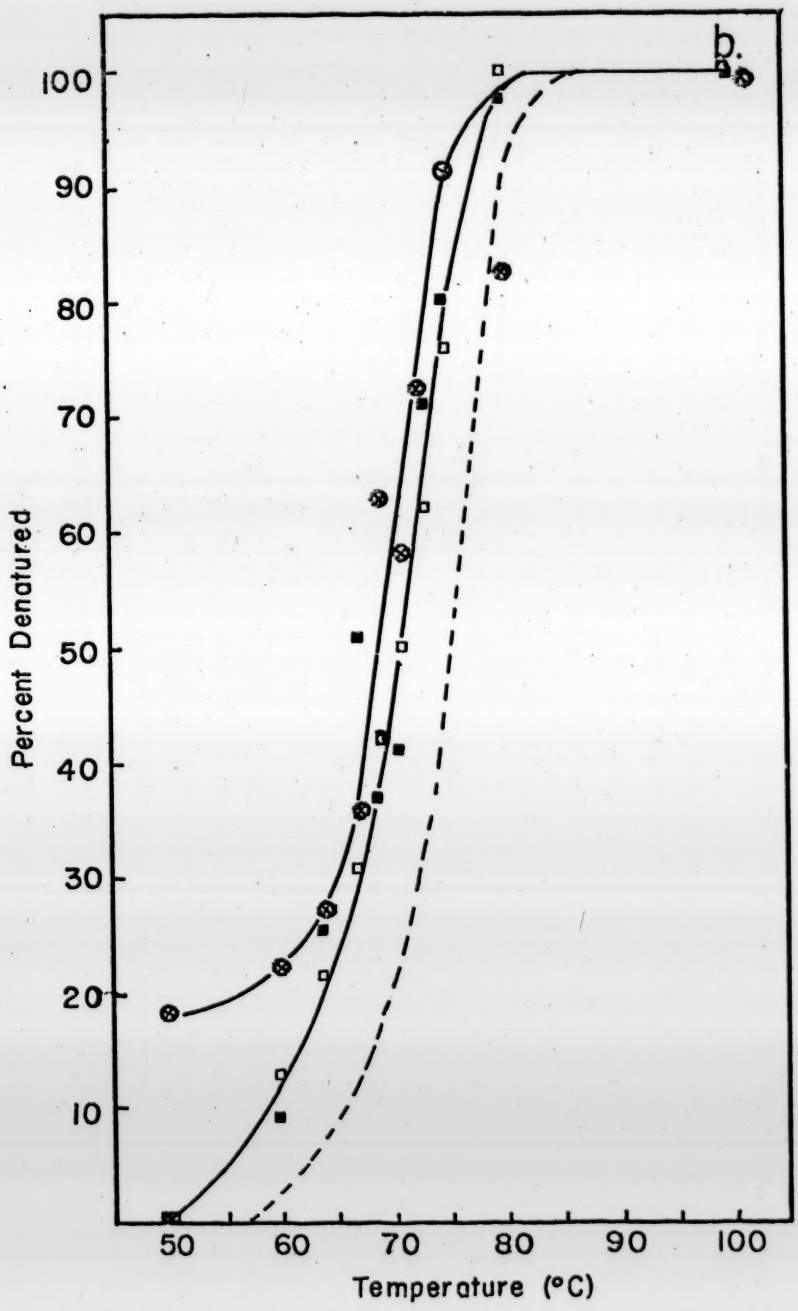
70
80
90
100
Temperature (°C)


b.
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Percent Denatured
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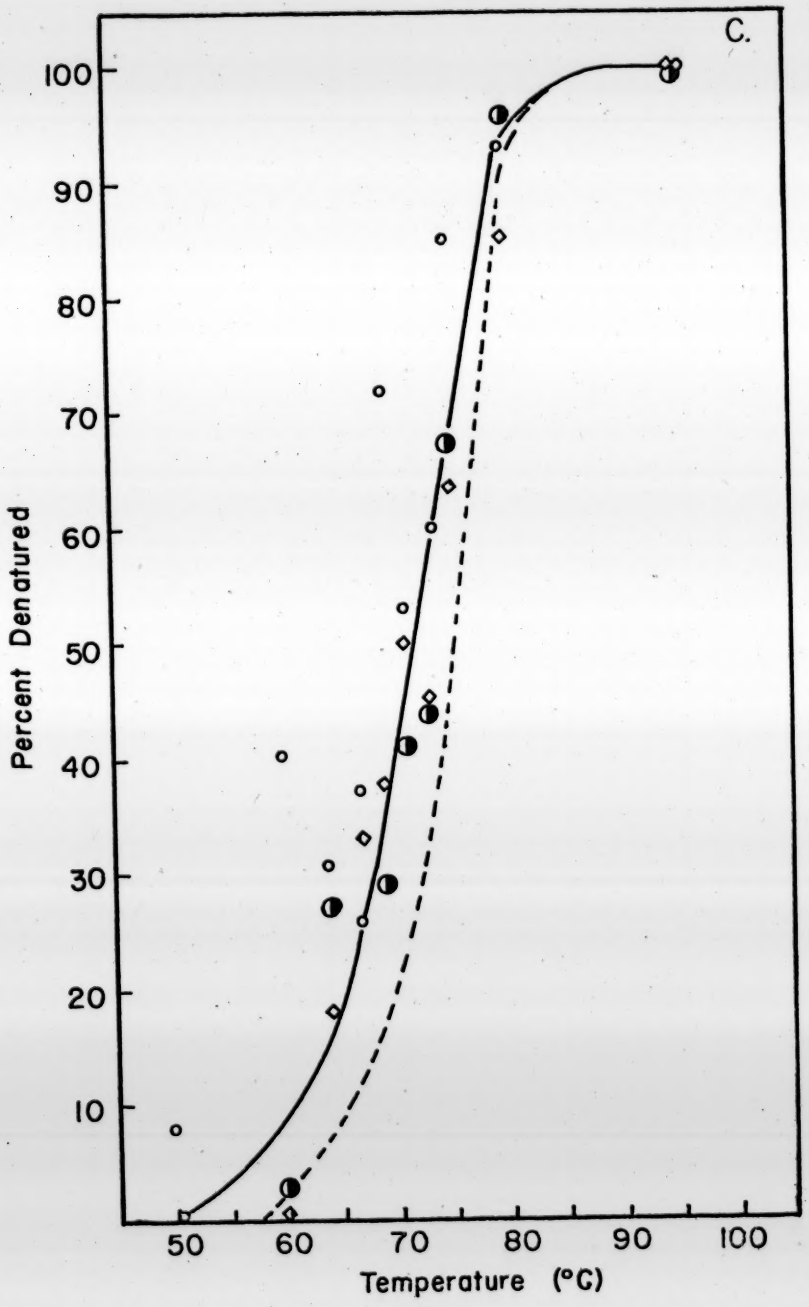
Temperature (°C)


C.
Percent Denatured
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80
70
60
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90
100
Temperature (°C)

result in a lowering of the Tm (Thomas and Dancis, 1973).

The percent of mismatching for each set of hybrids was calculated according to the estimates by Ullman and McCarthy (1973) for the effect of base pairing error upon the Tm and is listed in Table VI. The percent of mismatching ranged from 3-5%, indicating that the viral sequences present in mouse DNA may differ somewhat from strain to strain. This variation could be due to sequence divergence within the same MMTV genome or to the existence of different types of endogenous MMTV in each strain, as suggested by Bentvelzen (1972).

E. Hybridization of MMTV 70S RNA with Mouse DNA in DNA Excess:

It was determined in the previous sections (A-1,C) that all of the mouse strains under study contained mouse mammary tumor virus-specific sequences in their DNA. However, the degree of viral genomic representation in mouse DNA could not be assessed with MMTV DS-DNA or MMTV cDNA because of their limited representation of the viral genome. To examine the extent of MMTV gene representation in mouse DNA, MMTV 70S RNA was hybridized with mouse DNA in solution under conditions of DNA excess (Gelderman et al., 1968; Melli et al., 1971; Bishop, 1972).

This approach has the advantage of detecting a larger proportion of viral sequences in cell DNA, but it is also subject to a number of limitations (Bishop, 1972; Straus and Bonner, 1972). When RNA is hybridized with DNA in solution, viral RNA must compete with its homologous DNA strand for complementary DNA sequences.

TABLE VI

Thermal denaturation of hybrids formed between MMTV cDNA and DNA from various strains of mice

DNA	TM (°C)		change in Tm *	% mismatching **
	thermal elution HAP (0.12M PB)	S1 nuclease digestion (0.02M Tris)		
RIII	75.5	75.5	0	0
C3H	75.5	71.5	4	2.7
C3HeB/Fe	75.5	71.5	4	2.7
GR	75.5	64.4	8.1	5.4
C57BL/6	75.5	69.0	6.5	4.3
BALB/c	75.5	71.0	4.5	3.0
MMTV DS-DNA	----	72.5	3.0	---
"unique sequence" DNA	78.5	----	---	---

*Change in Tm = Tm for RIII hybrids - Tm for the other hybrids as assayed by S1 digestion.

**1% mismatching = 1.5°C change in Tm (Ullman and McCarthy, 1973).

Competition is impeded by the inefficency of RNA hybridization compared to DNA renaturation and is of greatest consequence when examining low frequency sequences in eukaryotic cells. The high Cot1/2 of renaturation and the lower rate constant for hybridization severely limit the extent of hybridization that can be achieved under attainable annealing conditions (see Discussion).

Despite these restrictions, differences in the extent of viral gene representation in eukaryotic DNA have been demonstrated by this technique (Hansen, 1972; Neiman, 1972; 1973; Neiman et al., 1974; Shoyab et al., 1974). With RSV, up to 70% of viral RNA could be hybridized to DNA from cells reportedly containing 2-4 copies of viral information per haploid genome (Neiman et al., 1974). Since earlier experiments had indicated that mouse cells may contain larger numbers of MMTV copies (Table II, VI), it was expected that MMTV RNA would also hybridize to at least 70% if all virus sequences were represented in cellular DNA.

MMTV 70S RNA was purified from virus released by explants of BALB/c f C3H or BALB/c mammary tumors and from a GR cell line originating from a spontaneous mammary tumor in a GR mouse. Virus produced by BALB/c f C3H explants will be called MMTV-S; by BALB/c explants, MMTV-O; and by GR cells, MMTV-P, according to the convention proposed by Bentvelzen (1972). From biological, immunological and genetic evidence it has been suggested that these viruses are different (see Introduction). It was hoped

that hybridization of MMTV RNA from three different sources with DNA from the various mouse strains might serve to clarify this issue.

1. Annealing of MMTV-S 70S RNA with Mouse DNA - MMTV-S 70S [^{32}P]RNA was obtained from BALB/c f C3H mammary tumor cells by growing tumor explants in media containing 1mCi/ml of [^{32}P]ortho-phosphate. Procedures for labeling and isolation of viral RNA have been described in detail elsewhere (Materials and Methods, I-1). BALB/c f C3H mammary tumor explants were demonstrated to be free of MuLV production by immunodiffusion (Teramoto et al., 1974) and radioimmuno-assay (Cardiff, personal communication). The exact specific activity could not be determined directly for MMTV-S 70S RNA as the recovery of viral RNA was below detection by optical means. Therefore, the specific activity of MMTV-S 70S RNA was estimated as being equal to the specific activity of ribosomal RNA (4.2×10^6 cpm/ug) extracted from tissue culture cells which had been used for producing virus.

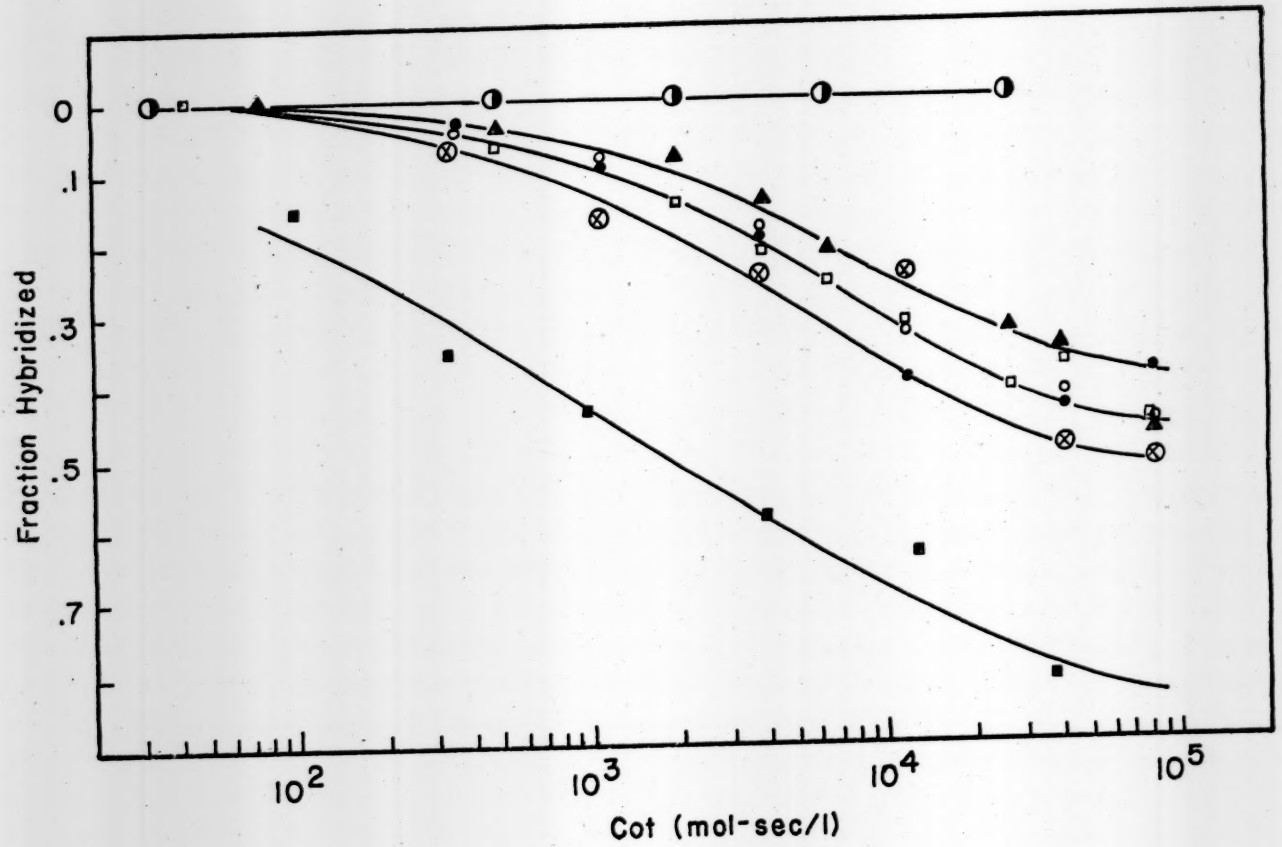
Mouse 18S ribosomal [^{32}P]RNA (10ng/ml; 8.3×10^5 cpm/ug) was hybridized with mouse DNA (10 mg/ml) in 0.4M PB, 68°C to determine the maximum extent of hybridization that could be expected under these annealing conditions. Ribosomal RNA was chosen because mammalian cells contain multiple copies of ribosomal genes (Bross and Krone, 1972) and an adequate sequence ratio of ribosomal DNA:ribosomal RNA is easily attained. Up to 80% of ribosomal RNA was annealed at a Cot value of 10^4 mol-sec/l without

appearing to reach a plateau (Figure 12). This corresponded to the results obtained by Melli et al. (1971) with rat ribosomal RNA, and indicated that a high percentage of [^{32}P]RNA was capable of hybridizing under the above conditions when provided with a sufficient excess of complementary DNA sequences.

MMTV-S 70S[^{32}P]RNA was hybridized with DNA from RIII, C3H, C3HeB/Fe, GR, C57BL/6 and BALB/c mice or with salmon sperm DNA in 0.4M PB at 68°C, at 10mg/ml of DNA, with an approximate DNA: RNA mass ratio of $1.5X10^6$ (Figure 12). Salmon sperm DNA served as a negative control and showed no hybridization with viral RNA. Hybridization of MMTV-S RNA and mouse DNA proceeded slowly with small differences in the rate and extent of annealing among the various mouse strains. At a Cot value of $8X10^4$ mol-sec/l, viral RNA was hybridized 40% with BALB/c DNA; 46% with RIII, C3H and C3HeB/Fe DNA; and 52% with GR DNA. This was the same pattern obtained with viral cDNA and suggests that GR DNA may contain more MMTV copies or a greater extent of viral information per genome than the other strains of mice. Alternatively, the slight differences in the rate and extent of reactions may reflect experimental variation. However, experiments with BALB/c and C3HeB/Fe DNA have been repeated with reproducible results. Furthermore, depending upon the initial sequence ratio (DNA:RNA) and the relative rates of hybridization and reassociation, a five fold difference in copy numbers could yield less than a 10% difference in the extent of hybridization (Straus and Bonner, 1972). Also, a 5-10% variance in the extent of hybridization can be indicative of

Figure 12. Hybridization of MMTV-S 70S RNA or ribosomal RNA with mouse cellular DNA.

MMTV-S 70S RNA (6.6 ng/ml, 4.15 X 10^6 cpm/µg) was annealed to mouse cellular DNA (10 mg/ml) or salmon sperm DNA (10 mg/ml), and GR cell 18S ribosomal [^{32}P]RNA (10.4 ng/ml, 8.3X10^5 cpm/µg) was hybridized to GR cellular DNA (10 mg/ml) in 0.4 M PB at 68°C. Samples (0.1 ml) were removed at various times into 2XSSC and assayed for resistance to pancreatic ribonuclease (50 µg/ml, 30 min 37°C). The fraction hybridized is plotted against Cot (mol-sec/l) for unlabeled cellular DNA on a semi-log scale. Hybridization of MMTV-S 70S RNA with salmon sperm DNA (◐), or with RIII (●), GR (⊗), C3H (□), C3HeB/Fe (o), C57BL/6 (△), or BALB/c (▲) DNA. Hybridization of ribosomal 18S RNA (■) to GR cellular DNA.



a genuine difference in the extent of genome representation in a particular DNA (Neiman et al., 1974). Because MMTV-S RNA did not hybridize to greater than 52%, it is difficult to determine whether incomplete annealing was a result of an inadequate DNA:RNA sequence ratio, or if MMTV-S RNA contained sequences which are absent from the mouse cell genome. It will be necessary to repeat these reactions using higher DNA:RNA ratios to clarify this issue.

2. Annealing of MMTV-P or MMTV-O 70S RNA with GR or BALB/c DNA - MMTV-P and MMTV-O were chosen to examine the extent of MMTV representation in mouse cell genomes for the following reason: If MMTV-P and MMTV-O are transmitted genetically in GR (MMTV-P) and BALB/c (MMTV-O) mice, then the DNA of these mice should contain all viral sequences at least once per haploid genome in the host mouse strain. Furthermore, with an adequate viral DNA:RNA sequence ratio one would expect at least 70% hybridization to host cell DNA.

MMTV-P 70S[^{32}P]RNA was obtained from GR cells and MMTV-O 70S[^{32}P]RNA from BALB/c mammary tumor explants by growing the cells in media containing 2 mCi/ml of [^{32}P]orthophosphate. Procedures for labeling and isolating viral RNA and for estimating specific activity of the RNA were the same as those described for MMTV-S RNA. Ringold et al. (1975) showed that GR cells were free of MuLV synthesis by hybridizing cDNA with nucleic acid isolated from the media of virus producing cell cultures.

Nucleic acid from these cells hybridized with MMTV cDNA and not with Ki-MuLV cDNA. Nucleic acid from the media of BALB/c mammary tumor explant cultures was not tested for MuLV sequences but did hybridize to 70% with MMTV cDNA without reaching a plateau. This suggests that viral RNA isolated from the media of BALB/c mammary tumor explants was predominately from MMTV but does not eliminate the possible presence of MuLV sequence in this nucleic acid.

Optimum conditions of hybridization were determined in preliminary experiments by annealing MMTV-P 70S [^{32}P]RNA (0.1 ng/ml; $8.6X10^6$cpm/ug) with GR DNA (10 mg/ml) in 0.4M PB or 4XSSC at 68°C and 75°C (Table VII). Similar hybridizations were performed in 50% formamide at 50°C (Table VII). Maximum hybridization (74%)

TABLE VII

The optimum conditions for hybridization of MMTV RNA to an excess of mouse DNA

Time	Cot		68°C*		75°C*		50°C, 50% Formamide*	
	0.4m PB	4XSSC	0.4M PB	4XSSC	0.4m PB	4XSSC	0.4M PB	4XSSC
12 hr.	$6.7X10^3$	$7.7X10^3$	--	--	48	50	--	--
24 hr.	$1.3X10^4$	$1.6X10^3$	38	64	53	56	50	46
72 hr.	$4.0X10^4$	$4.6X10^4$	59	73	--	--	62	65
102 hr.	$6.7X10^4$	$7.7X10^4$	58	74	--	--	69	68

*maximum percent resistance to digestion by pancreatic ribonuclease.

was achieved with 4XSSC at 68°C and was 15% greater than hybridization performed in 0.4M PB at the same temperature.

The DNA:RNA mass ratio necesary for obtaining the greatest extent of annealing was also examined using MMTV-P 70S RNA and GR DNA. Various amounts of MMTV-P 70S [^{32}P]RNA were hybridized with a standard amount of GR DNA at 10mg/ml of DNA in 4XSSC with 0.025% SDS at 68°C. Reactions were carried to a Cot of $2.7X10^4$ mol-sec/l and assayed for resistance to digestion by pancreatic ribonuclease (50 µg/ml, 37°C, 30 minutes). Results are given in Table VIII. Maximum hybridization was achieved at a DNA:RNA ratio of $2X10^6$. Increasing the ratio to $1X10^8$ (see above, Table VII) did not affect the extent of hybridization, while lower ratios yielded significant decreases in the final extent

TABLE VIII

The extent of hybridization of MMTV 70S RNA at different DNA:RNA mass ratios

Ratio	Average % hybridization
$2.0X10^6$	79
$7.0X10^5$	65
$4.2X10^5$	52
$2.0X10^5$	47
$1.4X10^5$	51
$8.3X10^4$	39

of annealing. All further hybridizations were performed at a mass ratio of $2X10^6$.

To determine the extent of viral sequence representation in GR DNA for each virus, MMTV-P or MMTV-O 70S [^{32}P]RNA (5ng/ml) was annealed under optimum hybridization conditions (4XSSC, 68°C, 0.025% SDS) with 10mg/ml of GR DNA (Figure 13a). MMTV-P and MMTV-O RNA hybridized with the same kinetics and to an equal extent (70%) with GR DNA, indicating that a majority of sequences for both viruses is present in GR cell DNA.

When MMTV-P or MMTV-O 70S [^{32}P]RNA (50ng/ml) was annealed with BALB/c DNA (10mg/ml), reproducible differences in the rate and extent of hybridization for each virus were consistantly obtained (Figure 13b). MMTV-P RNA hybridized slowly and reached a plateau of 42% annealing at a Cot of $8X10^4$ mol-sec/l, while MMTV-O RNA annealed slightly faster and plateaued with 54% hybridization at the same Cot value. These results suggest that sequence differences do exist between MMTV-P and MMTV-O viruses, since RNA containing identical sequences would be expected to anneal with similar kinetics to the same DNA.

At equal DNA:RNA mass ratios, neither viral RNA annealed to the same extent with BALB/c DNA as with GR DNA, implying that BALB/c DNA has fewer MMTV sequences than GR DNA. This has been a consistant finding using virus-specific nucleic acids from RIII, BALB/c f C3H, BALB/c or GR mice. As with MMTV-S RNA, it cannot be concluded whether the lower extent of hybridization

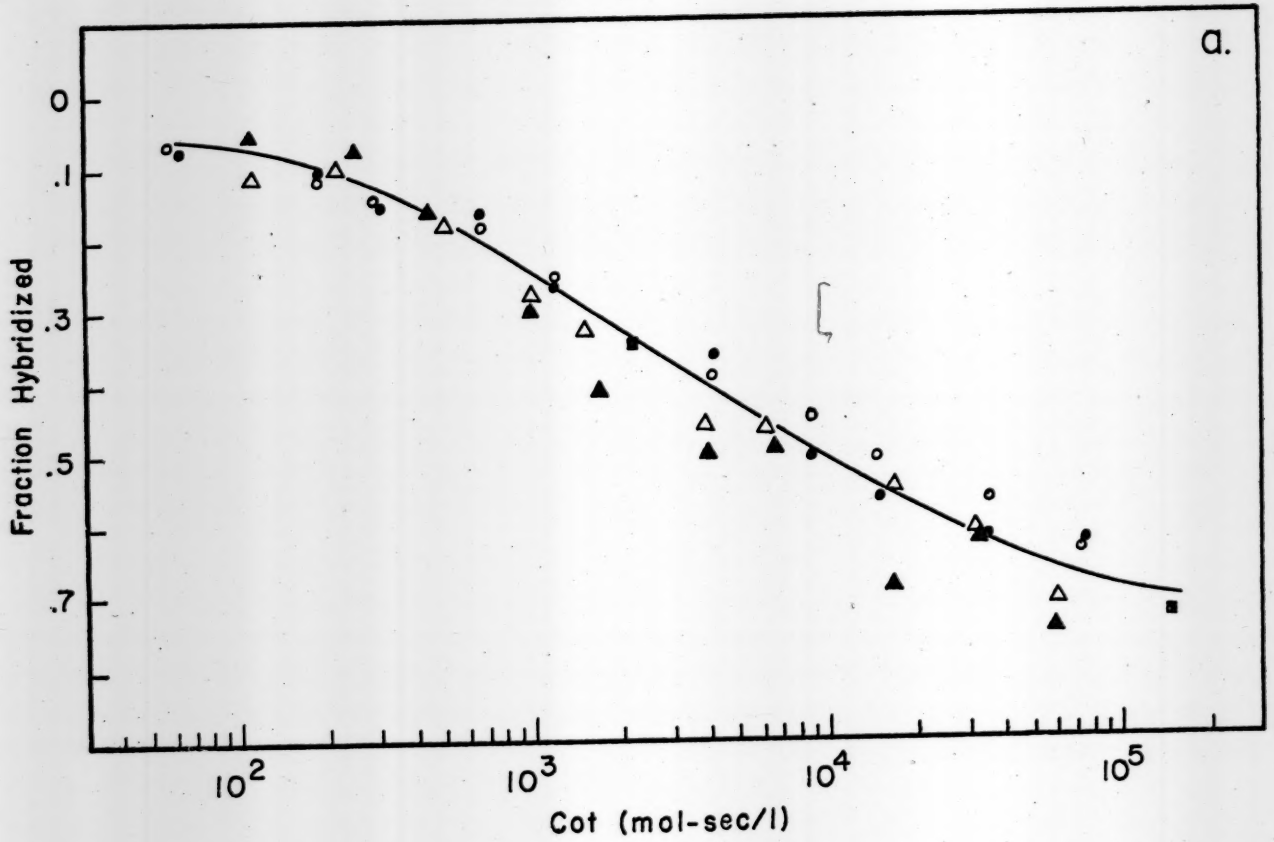
Figure 13. The hybridization of MMTV 70S RNA with GR or BALB/c cell DNA and with BALB/c mammary tumor DNA.

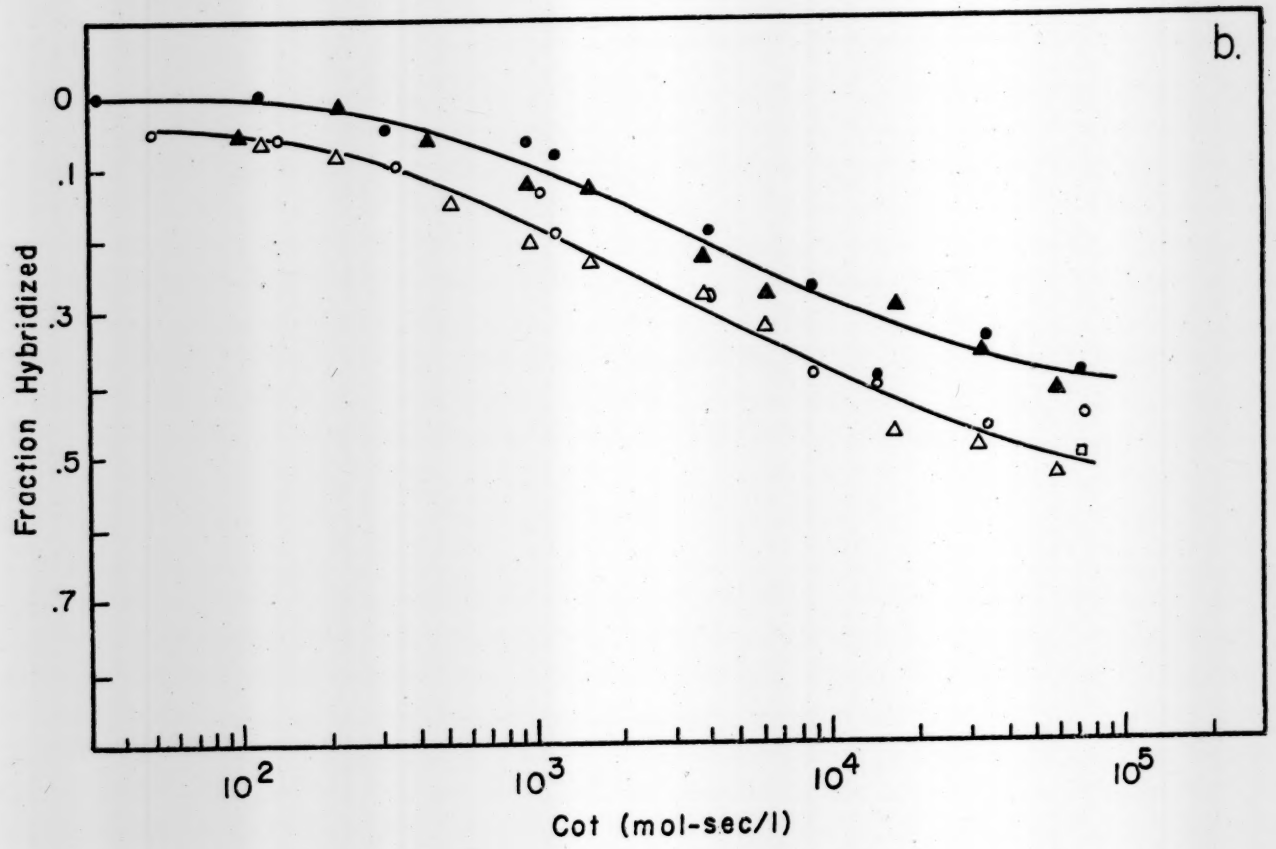
MMTV-P or MMTV-0 70S [^{32}P]RNA was annealed separately with GR or BALB/c cell DNA and with BALB/c mammary tumor DNA (4XSSC, 68°C, 6-10 mg/ml of DNA). All hybridizations were performed at a DNA:RNA mass ratio of $2X10^6$. Samples were removed at intervals, diluted into 2XSSC and assayed for resistance to pancreatic ribonuclease (50 µg/ml, 30°C, 30 minutes). The percent ribonuclease resistance has been corrected for the intrinsic ribonuclease resistance of unannealed 70S RNA (MMTV-P, 14%; MMTV-0, 12-25%), and is plotted against Cot (mol-sec/l) for cell DNA on a semi-log scale.

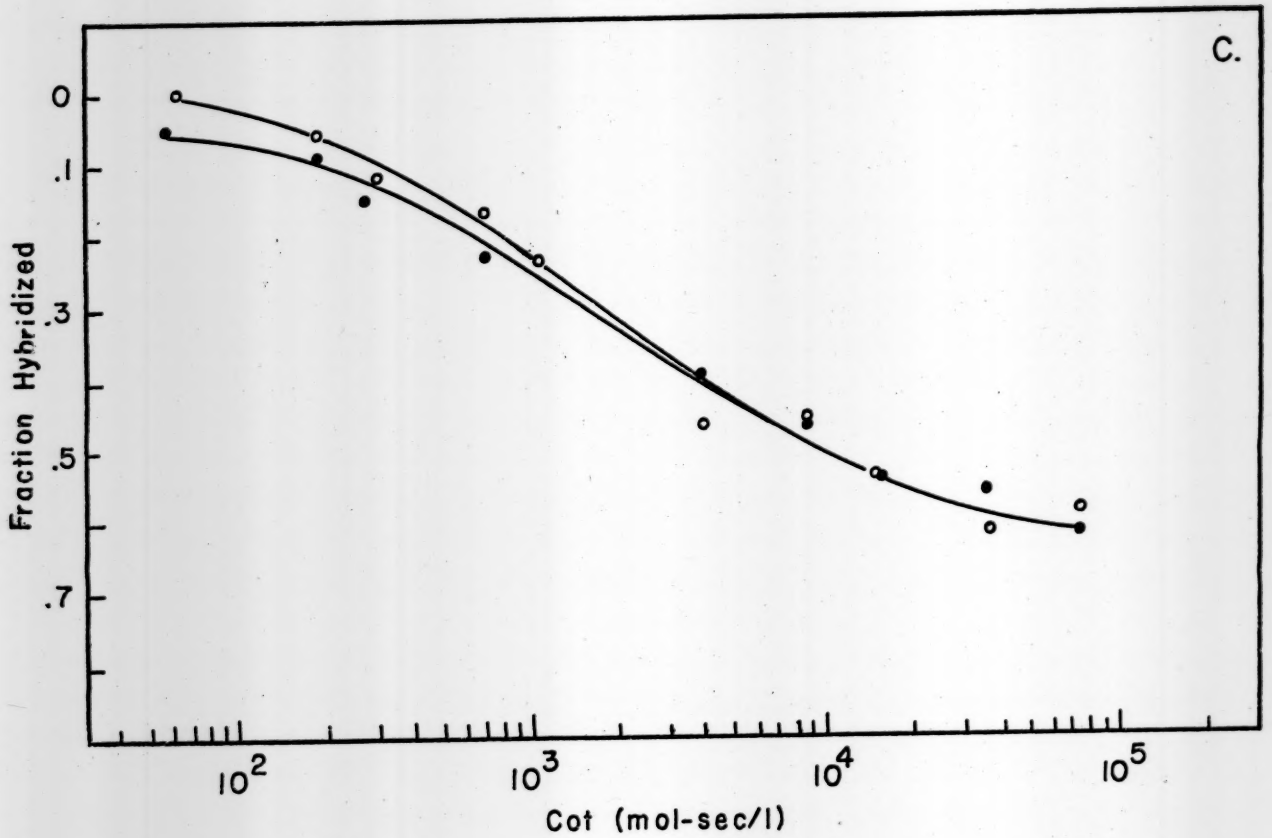
(a) Hybridization of MMTV-P RNA (●, ▲, ■) or MMTV-0 70S RNA (○, △) with GR cell DNA.

(b) Hybridization of MMTV-P 70S RNA (●, ▲), or MMTV-0 70S RNA (○, △, □) with BALB/c DNA.

(c) Hybridization of MMTV-P 70S RNA (●) or MMTV-0 70S RNA (○) with BALB/c mammary tumor cell DNA.







observed with MMTV-P and MMTV-0 RNA can be attributed to a lack of complete viral genomes. Since MMTV-0 was not isolated directly from a spontaneous mammary tumor, the possibility exists that complete MMTV-0 viral genomes may be present only in the European strain of BALB/c mice or in mammary tumors induced by MMTV-0.

3. Annealing of MMTV-P or MMTV-0 to 70S RNA with BALB/c mammary tumor cell DNA - European BALB/c mice were not available for testing, but BALB/c mammary tumor cell DNA was obtained from a BALB/c mammary umor produced by infection with MMTV-0. When MMTV-0 70S [^{32}P]RNA was hybridized with this DNA, it annealed with the same kinetics and to the same extent as it had with GR cell DNA (Figure 13c). MMTV-P 70S[^{32}P]RNA also hybridized rapidly and to the same extent with BALB/c mammary tumor DNA as with GR DNA (Figure 13c). However, MMTV-P RNA did not begin to anneal before a Cot of $6X10^{1}$ mol-sec/l. The extended annealing of MMTV-P and MMTV-0 70S RNA with BALB/c mammary tumor DNA suggests the presence of additional MMTV specific sequences which were not in normal BALB/c DNA.

Experiemnts with MMTV-S and MMTV-P or MMTV-0 RNA cannot be compared directly as hybridization conditions were not the same in each set of reactions. MMTV-S RNA was annealed in 0.4M PB, while MMTV-P and MMTV-0 RNA were annealed in 4XSSC. When these conditions were compared (Table VII), reactions in 4XSSC appeared to exceed those in 0.4M PB by approximately 15%. If one assumes that MMTV-S RNA would also show a 15% increase in the extent of annealing when hybridized in 4XSSC, then the final amount of

hybridization with GR and BALB/c DNA would be very close to that obtained with MMTV-0.

F. The Fidelity of Base Pairing Between MMTV 70S RNA and Mouse Cell DNA:

The fidelity of MMTV RNA hybrids was examined by thermal denaturation. MMTV-P 70S [^{32}P]PRNA was hybridized separately with GR and BALB/c DNA under maximum annealing conditions (see above). Nucleic acids were precipitated from solution, resuspended in 0.01M Tris:HCl, pH 8.1, samples heated to various temperatures, cooled immediately and assayed for resistance to digestion by ribonuclease (for detailed procedure see Materials and Methods).

TABLE IX

The mean thermal denaturation temperature for hybrids formed between MMTV 70S RNA and mouse DNA

RNA	DNA		
	GR	BALB/c	MMTV cDNA
MMTV-P	66.5	67.5	--
MMTV-P	67.0	69.0	--
MMTV* RIII milk virus	----	----	68

*Varmus et al., 1973b.

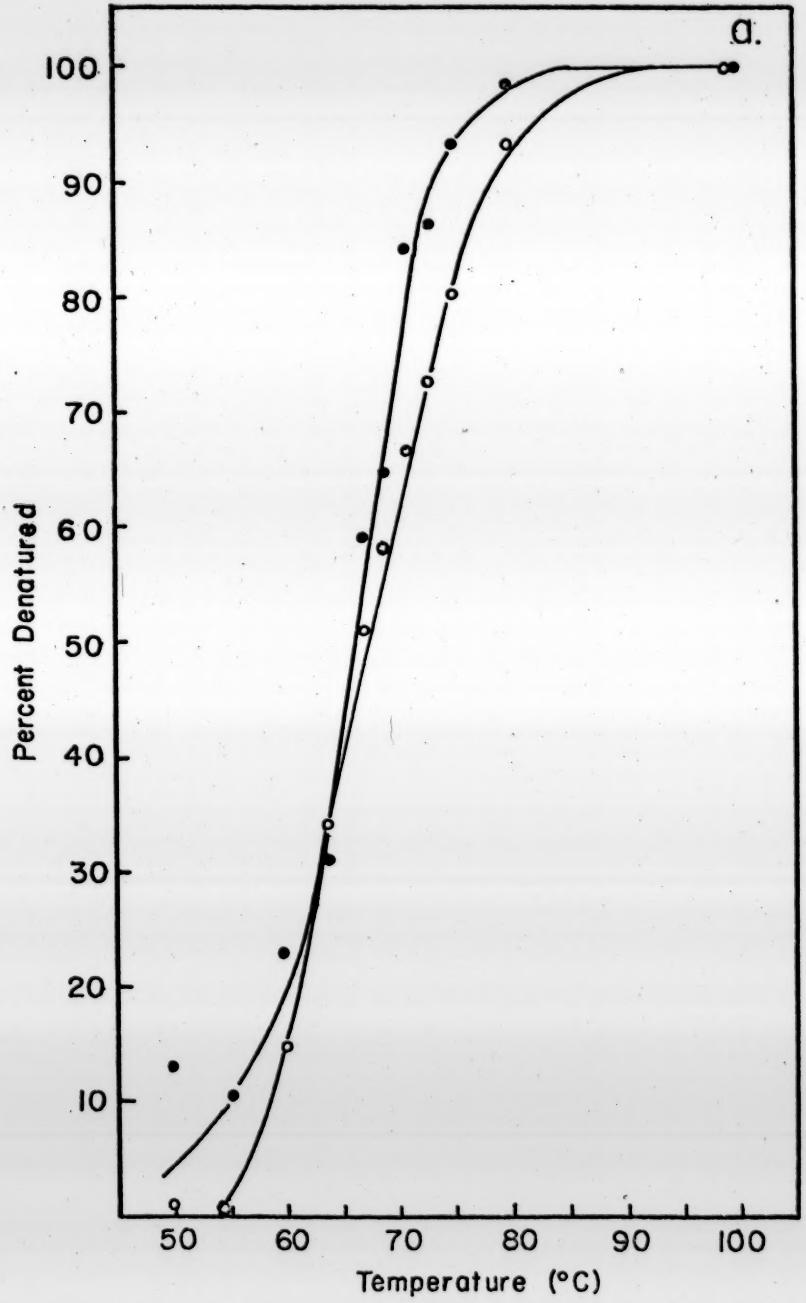
There was no significant difference in the mean thermal denaturation temperatures (Tm) for hybrids formed between MMTV-P or MMTV-O RNA and GR or BALB/c DNA (Table IX). All of the hybrids melted with approximately the same Tm (68°C) reported for MMTV 70S RNA which had been annealed to MMTV cDNA and denatured under identical conditions (Varmus et al., 1973b). This would imply that only MMTV specific hybrids had been formed with mouse cell DNA. Although the Tm of all hybrids was very similar, there was some difference in the general shape of the denaturation curves (Figure 14a,b). Hybrids formed between MMTV-P or MMTV-O RNA and GR DNA had a sharp denaturation profile, suggesting a population of hybrids with similar thermal stability. Duplexes formed between MMTV-P or MMTV-O and BALB/c DNA denatured with a broader profile, indicating greater heterogeneity in the stability of these hybrids.

Figure 14. Thermal denaturation of hybrids formed between MMTV [^{32}P] RNA and mouse cellular DNA.

MMTV 70S [^{32}P]RNA was hybridized to mouse cellular DNA at a DNA:RNA ratio of $2X10^6$ at 68°C in 4XSSC. Reactions were carried to a Cot value of $4X10^6$ mol-sec/l, ethanol precipitated and nucleic acids were resuspended in 0.01 M Tris:HCl, pH 8.1. Samples from each reaction were heated for fifteen minutes at the desired temperature and immediately cooled in an ice water bath. Each sample was assayed for susceptibility to digestion by pancreatic ribonuclease (50 µg/ml, 30 minutes, 2XSSC, 37°C). Results have been corrected for the percentage of unhybridized [^{32}P]RNA before denaturation (20-40%) and are illustrated as the percent denatured versus the temperature.

(a) Thermal denaturation of hybrids formed between MMTV-P 70S [^{32}P]RNA and GR (●) or BALB/c (o) DNA.

(b) Thermal denaturation of hybrids formed between MMTV-O 70S [^{32}P]RNA and GR (●) or BALB/c (o) DNA.


a.
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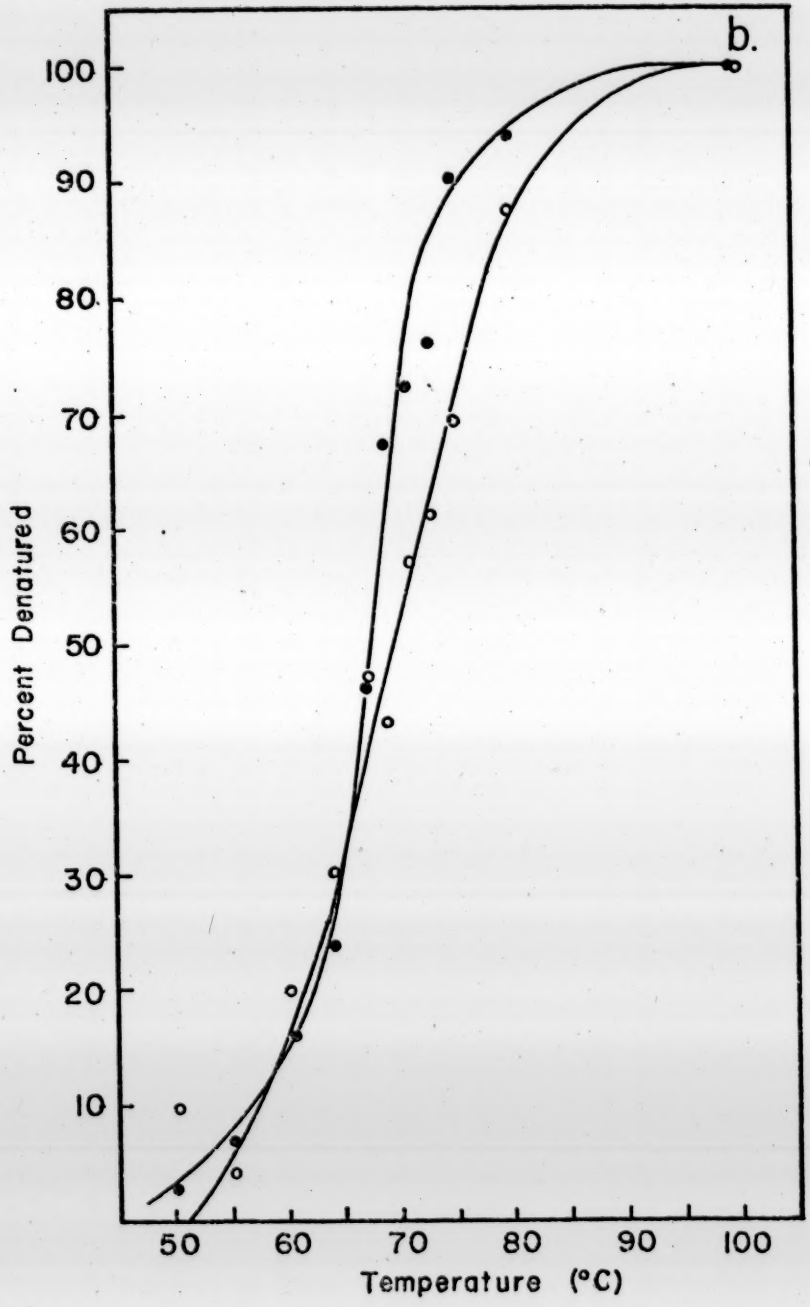
Temperature (°C)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DISCUSSION

I. Evaluation of Hybridization Techniques.

Three methods of molecular hybridization have been used to examine mouse cell DNA for mammary tumor virus-specific sequences. The first two methods involved the use of DNA transcripts synthesized by virion-associated, RNA-directed DNA polymerase from RIII milk mammary tumor virus. The third method utilized mammary tumor virus RNA obtained from mouse mammary tumor cells in culture. Each of these techniques has certain limitations which must be taken into consideration when evaluating experimental results. These limitations are discussed below as they pertain to the work presented in this dissertation.

A. Detection of MMT Virus-Specific Sequences in Mouse Cell DNA by Accelerated Reassociation of MMT Viral Double Stranded DNA:

The use of accelerated reassociation kinetics to evaluate the number of viral genome equivalents in cellular DNA was established by Gelb, Kohne and Martin (1971) for the assessment of SV 40 DNA in normal and transformed cells. This approach has been extended to RNA tumor viruses by following the reassociation of radioactively labeled virus-specific double stranded DNA, synthesized by viral RNA-directed DNA polymerase (Gelb et al., 1971a; Gelb et al., 1973; Varmus et al., 1971; Varmus et al., 1972a; Fujinaga et al., 1973). This technique has the advantages of providing reproducible viral copy numbers while requiring relatively small quantities of DNA. Its principal disadvantage is the uncertainty of absolute copy numbers based upon

indirect determination of the molecular complexity[1] and the concentration of viral DS-DNA. Furthermore, the incomplete genome representation of viral DS-DNA makes this technique insensitive for the detection of partial genome deficits.

When DNA from a virus such as SV 40 is used to detect viral DNA in cells, one assumes that the entire genome is composed of unique sequences, and therefore its complexity can be determined by its molecular weight. This relationship is not applicable to the DS-DNA transcripts of RNA tumor viruses as the Cot 1/2 for reassociation of DS-DNA is not compatable with a total unique sequence length equal to its molecular weight ($7X10^4$ daltons, Taylor et al., 1972). Consequently, the complexity of DS-DNA has been estimated by comparing its Cot 1/2 of reassociation with the Cot 1/2 of reassociaton of a standard DNA molecule of known complexity (Britten and Kohne, 1968). The complexity of MMTV DS-DNA was estimated to equal a total unique sequence length weighing $1.2X10^6$ daltons. This estimate was obtained by a direct proportionality drawn between the Cot 1/2 of reassociation for MMTV DS-DNA ($2.X\ 10^{-3}$ mol-sec/l, Figure 4) and the published Cot 1/2 and molecular weight for lambda phage ($6\text{-}7X10^{-2}$ mol-sec/l, $2.6X10^7$ daltons) and the fd phage replicative intermediate ($6\text{-}7X10^{-3}$ mol-sec/l, $3\text{-}4.4X10^6$ daltons; Varmus et al., 1971). A value of $1.2X10^6$ daltons compares nicely with earlier reports of $1X10^6$ daltons for MMTV

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[1] Complexity = the total number of DNA base pairs in nonrepeating sequences. In the case of virus the complexity is the number of base pairs in the viral chromosome (Wetmur and Davison, 1968).

DS-DNA and RSV rapidly reassociating (rr) DS-DNA (Varmus et al., 1972a; Varmus et al., 1971). A higher complexity (equal to $6X10^6$ daltons) has been reported for a minor population of RSV DS-DNA (slowly reassociating (sr) DS-DNA (Varmus et al., 1971), and complexities equal to $6X10^6$ daltons (rr) and $19X10^6$ daltons (sr) have been estimated for Ki-MuLV DS-DNA (Gelb et al., 1971a). The larger estimates obtained for MULV than for MMTV or RSV may be attributable to the different viral DNA standards used by the two laboratories when determining DNA complexity.

Although the complexity of DS-DNA appears to be greater than indicated by its size, it is still less than expected for complex representation of the viral genome. The major nucleic acid of RNA tumor viruses is an RNA molecule which has a sedimentation coefficient of 70S (approximately $1X10^7$ daltons) and is composed of three to four hydrogen bonded 30-40S subunits (approximately $3.5X10^6$ daltons; see review by Temin, 1971a). If the subunits contain unique sequences, the viral genomes would be haploid and would have a double stranded complexity equal to $2X10^7$ daltons. If they are identical or nearly identical, the viral genome would be polyploid with a double stranded complexity equal to approximately $7X10^6$ daltons. Evidence to favor a haploid genome has been provided by nucleic acid hybridization experiments (Taylor et al., 1974; Fan and Paskind, 1974), while data obtained by oligonucleotide finger printing (Beeman et al., 1974) would indicate polyploidy. In either case, the MMTV DS-DNA transcripts would appear to represent only 5-14% of the viral genome. Greater representation has been suggested for slowly reassociating (sr) fractions of MuLV

and RSV DS-DNA (Gelb et al., 1971a; Varmus et al., 1971). Bishop et al. (1973) were able to demonstrate 30% representation of viral sequences in DS-DNA by hybridizing RSV sr DS-DNA with RSV 70S RNA. This would be consistant with the estimated complexity of $6X10^6$ for RSV sr DS-DNA if viral 70S is composed of a haploid genome. However, the complexity of sr DS-DNA would be overestimated, should the virus contain a polyploid genome. The inconsistancy of the second possibility raises doubt concerning the complexity of other viral DS-DNA determined by comparative reassociation kinetics. Changes in the estimated complexity for DS-DNA would affect the determination of copy numbers (See Appendix).

Further uncertainty in the absolute copy numbers determined by accelerated reassociation kinetics results from the necessity to estimate the specific activity of viral DS-DNA, as there is not enough DNA to determine its concentration optically. Any mistakes in these estimates would give an incorrect value for viral/cellular DNA ratio when calculating copy numbers.

In view of the uncertainties surrounding the complexity of viral DS-DNA molecules, their estimated specific activities and their limited genome representation, the accuracy of viral copy numbers obtained by the accelerated reassociation of DS-DNA is questionable.

B. Quantitation of MMT Viral Genes in Mouse Cell DNA by Hybridization with MMTV cDNA:

The second approach used to detect and to quantify MMT virus specific sequences in cellular DNA from high and low incidence mouse strains was to follow the reassociation of viral sequences with small

amounts of highly-labeled single-stranded, complementary viral DNA (cDNA) which has been synthesized by detergent activated, virion-associated, RNA-dependent DNA polymerase in the presence of actinomycin D. This approach has two major advantages over determinations made by accelerated reassociation of DS-DNA. First, cDNA is generally a more representative viral transcript than DS-DNA and therefore is capable of detecting a large proportion of viral sequences. Secondly, viral copy numbers can be more accurately estimated by following the rate of annealing for cDNA in relationship to the reassociation rate for "unique sequence" cellular DNA. The principal disadvantages of this method are that large quantities of cell DNA are required for complete hybridization curves, and the maximum extent of hybridization is generally achieved when only 60-70% of the cDNA has been annealed. This complicates the calculations of copy numbers and raises the possibility of incomplete representation of viral sequences in cellular DNA.

Limited hybridization (60-70%) of MMTV cDNA was obtained when it was annealed with DNA from any of the six mouse strains under study (Figure 8a-f); analogous results have been reported for MMTV cDNA by others (Parks and Scolnick, 1973). Similar extents of hybridization have also been observed with complementary DNA transcribed from RSV RNA (Varmus et al., 1974b), mouse immunoglobulin L chain messenger RNA (Stavnezer et al., 1974), chicken ovalbumin messenger RNA (Sullivan et al., 1973) and MuLV RNA (Viola and White, 1973). While 80% hybridization has been documented for cDNA transcribed from MuLV, wolly monkey sarcoma virus and gibbon lympho-sarcoma virus (Scolnick et al., 1974), no one has obtained

complete hybridization of viral cDNA to cellular DNA[1]. The incomplete annealing of cDNA could possibly be attributed to one or more of the following: (1) A fraction of cDNA may not be capable of hybridization. (2) Some sequences may be represented in cDNA which are not present in the cellular DNA. (3) There may be single stranded "tails" which are susceptable to S1 nuclease digestion. (4) There may be an insufficient excess of unlabeled viral sequences in the cell DNA. (5) There may be ineffective competition between labeled and unlabeled viral sequences. Each of these possibilities is considered separately below with respect to MMTV cDNA.

Does cDNA contain some molecules which are unable to hybridize? Chattapadhyay et al. (1974) were able to show that a portion (20%) of their MuLV cDNA was not capable of hybridization with MuLV RNA, RNA from MuLV producing tissues or with DNA from MuLV infected cells. However, MMTV cDNA which was not able to anneal greater than 70% with cellular DNA could hybridize completely with RNA from MMTV producing tissues (Figure 6). This suggests that DNA from most MMTV cDNA preparations is capable of complete hybridization if provided with a sufficient excess of complete gene sequences.

Does MMTV cDNA contain sequences which were not represented in the mouse cell DNA? With RSV and MuLV, at least a portion of cDNA

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[1] One report of complete hybridization of cDNA with cellular DNA has appeared in the literature (Schincariol and Joklik, 1973); however these data represented corrected values (Schincariol, personal communication).

which did not react with DNA from uninfected cells was capable of hybridizing with DNA from infected virus producing cells (Varmus, unpublished observations; Viola and White, 1973). This indcates that in some cases there are additional sequences in DNA from infected cells which may be absent in normal cells. In the present experiments, DNA was obtained from normal adult male mice or unsexed newborns and not from virus producing mammary glands or mammary tumors. Thus raising the possibility that MMTV cDNA did not anneal to completion because normal tissues did not contain complete viral information. Since non-annealing MMTV cDNA was not isolated and hybridized with DNA from virus producing tissues, this possibility cannot be entirely eliminated. There is evidence, however, suggesting that adult male tissues do contain complete viral information. MMTV can be transmitted by spleen or blood cells from "normal" male mice which have been naturally infected at birth through nursing (Nandi et al., 1974). It can be transmitted in seminal fluid (see review, Nandi and McGrath, 1973). And genetic experiments have provided evidence to suggest that many strains of mice have complete viral information which is transmitted through their gametes (Bentvelzen, 1972). If all mice do contain complete MMTV specific sequences in their DNA, these sequences need not be completely homologous between mouse strains. Genetic variations in MMTV have been indicated by obvious differences in morphology among mammary tumors occurring after infection by RIII milk virus and those which develop spontaneously or following infection with virus from other mouse strains (see review, Nandi and McGrath, 1973). Since all

MMTV cDNA was prepared with RIII milk virus, this cDNA may share some but not all sequences with MMTV endogenous virus(es). Some support of this hypothesis is provided by the variation in denaturation profiles obtained for hybrids formed between RIII MMTV cDNA and cellular DNA from each mouse strain (Figure 11). This explanation does not account for the incomplete hybridization of RIII cDNA with RIII DNA. Whether un-hybridized RIII cDNA can anneal with DNA from RIII producing mammary glands or mammary tumors remains to be determined.

Do MMTV cDNA hybrids contain single stranded "tails" which are digested by S1 nuclease? When annealing of MMTV cDNA is assayed by hydroxylapetite fractionation or by digestion with S1 nuclease, a higher percentage of hybridization (70%) is obtained by HAP than by S1 (56%, unpublished observation). This difference can be attributed to the presence of single stranded "tails" on hybrid molecules which are detected by S1 digestion and not by HAP fractionation. The presence of such "tails", however, can not account for the remaining 30% of un-hybridized MMTV cDNA.

Is there an insufficient amount of certain viral sequences in cell DNA to allow complete hybridization of cDNA? Gradual increases in the extent of annealing were achieved by the sequential hybridization of cell DNA with MMTV cDNA. But complete hybridization of 0.12 ng of cDNA could not be obtained with 6 mg of cell DNA. Therefore, at a DNA/RNA mass ratio of $5X10^7$ mouse cell DNA did not have an adequate complement of viral sequences. This result can be explained by the presence of very redundant sequences in cDNA. Preferential transcription of

viral DNA is indicated from the RNA/DNA ratio (1/15) necessary to obtain maximum hybridization (60%) of cDNA with viral RNA (Figure 5). The degree of preferential transcription may be underestimated in Figure 5, as the ratios rely upon the specific activity of viral RNA, and the specific activity for MMTV RNA was considered equal to ribosomal RNA isolated from radioactively labeled virus producing cells. RSV RNA obtained from cells labeled in a similar fashion had twice the specific activity of ribosomal RNA (Neiman, unpublished observation). The same relationship applied to MMTV RNA would increase the RNA/DNA ratio two fold (1/30). Heterogeneity in the sequence representation of cDNA was also implied from the kinetics of hybridization of MMTV cDNA with mouse cell DNA in Figure 9a,b,c. However, from these data it is not clear if the heterogeneity lies in the cDNA or cell sequences.

Is there effective competition between MMTV cDNA and viral sequences present in cellular DNA? When MMTV cDNA was reacted with C3HeB/Fe cell DNA to a Cot of $2.7X10^4$ mol-sec/l at a DNA/RNA ratio of $2X10^7$, it generally hybridized to 60%. If the annealed DNA was isolated from one of these reactions by fractionation of hydroxylapatite, denatured and reassociated, only 60% of the MMTV cDNA, which had previously been hybridized, was reannealed. Similar observations for MuLV cDNA have been reported. In this case 80% hybridization as assayed by HAP was the maximum amount of hybridization obtainable at any time (Chattapadhyay et al., 1974; Lowy, personal communication). These results are indicative of ineffective competition between cDNA and its homologous cell DNA strand for complementary DNA sequences. Variations in viral sequences between the endogenous MMTV in each mouse strain and the

RIII milk virus could be responsible for this ineffective competition, since the annealing rate is impeded if the sequences involved are not identical. At stringent hybridization conditions the reassociation of homologous sequences occurs at a greater rate than heterologous sequences and can be completed before all heterologous DNA can hybridize (McCarthy and Farquhar, 1973). The size of cDNA may also impede its hybridization with viral sequences in cellular DNA since the hybridization rate is controlled by the size of smallest reactant (Lee and Wetmur, 1972). RSV cDNA is heterogeneous in size with the major forms being 50-100 nucleotides in length (Taylor et al., 1972). If MMTV cDNA is similar in size, it would be expected to react more slowly with complementary seqences in cellular DNA fragments than homologous cell DNA strands (approximately 150-200 nucleotides long) (Varmus et al., 1973c).

It would appear that the incomplete hybridization of MMTV cDNA can be attributed to a combination of factors, the most important being those affecting the kinetics of hybridization and the possible incomplete representation of RIII milk virus sequences in cell DNA. The kinetics of hybridization of RNA:DNA insolution are discussed below and pertain to viral cDNA:cell DNA hybridization as well. The preferential transcription of certain segments of the viral genome causes a variation in the initial sequences ratios for virus specific sequences and affects the rate and extent of hybridization. The sequence divergence between MMTV variants and possible heterogeneity in the size of viral transcripts in cDNA also affects the rate of hybridization and therefore the shape and extent of the hybridization curves. Because copy numbers are computed

from the rate of hybridization and the rate is determined by a number of parameters other than the frequency of viral sequences in cell DNA, there is some uncertainty concerning the absolute copy number obtained by this technique.

C. Hybridization of MMTV 70S RNA with Mouse Cell DNA in DNA Excess:

To evaluate the degree of viral gene representation in mouse cell DNA, MMTV RNA was hybridized directly with mouse DNA in solution under conditions of DNA excess (Melli et al., 1971; Gelderman et al., 1971). The principle of this technique is to monitor the reassociation of virus specific sequences in cellular DNA by following the hybridization of small amounts of highly labeled viral RNA. RNA:DNA hybridization in solution permits the detection of viral sequences which may be present at a low frequency in cellular DNA. The use of viral RNA provides a complete and uniform probe for such viral sequences. Since confirmation of total viral genome representation in cell DNA relies upon the complete hybridization of RNA, a potential disadvantage of this technique results from the inability of RNA to hybridize much more than 70% with cell DNA at Cot values which are practical for extensive analysis. A brief review of some theoretical considerations for the annealing of RNA with DNA in solution may clarify a few of the problems encountered with this type of reaction and aid in the interpretation of incomplete hybridization results. For a complete discusison involving the theoretical aspects of RNA:DNA hybridization in solution the reader is referred to Bishop, 1972; Straus and Bonner, 1972; Tereba, 1973.

Equations describing the reassociation of cell DNA and hybridization of RNA in solution are given below:

$$D_1 + D_2 \underset{-k^D}{\overset{k^D}{\rightleftharpoons}} D_1D_2 \qquad (1)$$

$$R + D_2 \underset{-k^R}{\overset{k^R}{\rightleftharpoons}} RD_2 \qquad (2)$$

D_1 and D_2 represent the concentration of the two strands of DNA; D_2 being the strand which is complementary to RNA; R the concentration of RNA; k^D the rate constant for the reassociation of DNA; k^R the rate constant for the hybridization of RNA; and $-k^D$ and $-k^R$ the respective reverse rate constants. If one assumes that these reactions proceed with second order kinetics, then equations for the disappearance of each reacting species can be derived from (1) and (2) above as follows[1]:

$$\frac{dD_1}{dt} = -k^D D_1D_2 \qquad (3)$$

$$\frac{dD_2}{dt} = -k^D D_1D_2 - k^R RD_2 \qquad (4)$$

$$\frac{dR}{dt} = -k^R RD_2 \qquad (5)$$

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[1] The reverse reactions are considered negligible under normal hybridization conditions.

When D_2 is in adequate excess (an initial sequence ratio of DNA/RNA greater than 70; Bishop, 1972), the concentration of D_2 will not be greatly affected by hybridization with RNA and equation (4) can be neglected. In such cases the equations can be solved analytically. But when an adequate sequence ratio is not achieved, these equations must be solved numerically. This can be done by measuring changes in R, D_1 and D_2 over small increments of time and analyzing the results by computer. When data for various values are expressed as the extent of reaction versus log Cot, the shape and extent of hybridization curves depend upon the ratio of the inital concentration of RNA to the initial concentration of either DNA strand (R_o) and k^R/k^D (Straus and Bonner, 1972).

Because viral genes are generally represented at a low frequency in cellular DNA and are diluted considerably by non-viral sequences, attempts to minimize R_o were made by reacting MMTV RNA with mouse cell DNA at a DNA/RNA mass ratio of $2X10^6$. (When the mass ratio was increased fifty fold, the final extent of hybridization remained unchanged; Table VII). However, even at these concentrations it is often difficult to achieve more than a moderate DNA/RNA sequence ratio.

An effective sequence ratio can be estimated for MMTV by assuming one complete MMTV genome per haploid mouse cell. With a molecular weight of $1X10^7$ daltons for viral RNA (Duesberg and Blair, 1966) and $2X10^{12}$ daltons for the haploid mouse genome at a mass ratio of $2X10^6$, there would be an effective sequence ratio of 10/1. The minimum number of viral copies/haploid mouse genome was estimated at five and the maximum

at thirty-two (Table V). Consequently, the effective sequence ratio should be increased to range from 50-320/1, depending upon which type of mouse DNA was being analyzed[1]. Indeed, when the same mass ratio was used for hybridization of MMTV-S with each of the mouse strains, the extent of hybridization observed varied relative to the predicted effective sequence ratios, i.e., GR > RIII, C3H, C3HeB/Fe > BALB/c (Figure 12).

With the initial sequence ratio held constant, the major factor influencing the extent of hybridization is the k^R/k^D ratio. When RNA is hybridized with cell DNA in solution, it must compete with its homologous DNA strand (D_1) for complementary DNA sequences (D_2). Since the concentrations of D_2 is less than the combined concentrations of R and D_1, neither hybridization nor reassociation can be expected to reach completion. Furthermore, it has been well documented that DNA:DNA reactions are more efficient than RNA:DNA reactions (Melli et al., 1971; Bishop, 1972; Straus and Bonner, 1972). Consequently, as k^R/k^D decreases there is a decrease in the amount of RNA hybridizing and a corresponding increase in the amount of DNA reassociated.

The rate constants for hybridization and reassociation are influenced by reaction temperatures and the size of the reacting polynucleotides. Higher temperatures can favor k^R as the optimum temperature for hybridization is approximately 10°C above the optimum for reassociation

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[1] These rates are based upon assumptions that MMTV has a haploid genome and that SA estimates for [^{32}P]-viral RNA were correct. A complexity of $3X10^6$ for viral DNA and a two fold increase in the viral DNA SA would decrease the effective ratio to 30-102/1.

(Bishop, 1972). The effect of temperature on hybridization has been attributed to possible secondary structure of RNA molecules at lower temperatures which could interfere with the annealing process (Strauss and Bonner, 1972). Since hybridization of MMTV-S RNA at 68°C did not exceed 50%, the effect of temperature was tested for annealing with MMTV-P RNA. Hybridization was improved at 75°C over 68°C in phosphate buffer. However, hybridization in 4 X SSC at 68°C was greater than at 75°C or in phosphate buffer at either temperature (Table VII). Therefore, optimal hybridization of MMTV RNA occurs at the same optimal temperature for reassociation of cell DNA.

The rate of DNA hybridization is a function of the square root of its chain length (Hutton and Wetmur, 1973). As the size of the RNA becomes smaller, there is a decrease in its k^R. High temperatures normally used in hybridization reactions to obtain specificity and the succeptibility of RNA to thermal scission can result in a progressive decrease in the size of RNA molecules and in the k^R. To avoid the effect of thermal scission, RNA:DNA hybridization is often performed in formamide at reduced temperatures (McConaughy et al., 1969). When MMTV-P RNA was hybridized with GR DNA in 50% formamide, 4 X SSC, at 50°C, the extent of hybridization was slightly less (6%) than at 68°C without formamide (Table VII). Therefore, the maximum extent of hybridization with MMTV was obtained at elevated temperatures despite the possible effect of thermal scission on RNA.

The extent of hybridization of RNA can be much less than 100% depending upon the R_o and k^R/k^D ratio. In addition, certain non-kinetic

factors can contribute to incomplete hybridization of RNA such as the presence of unhybridized ribonuclease sensitive "tails", sensitivity of hybridized RNA to digestion by ribonuclease and the presence of sequences in RNA which are not represented in cell DNA. The contribution of RNA "tails" to incomplete hybridization has not been assessed. But some evidence implicating limited ribonuclease digestion of hybridized RNA has been provided by the treatment of hybrids with single strand specific S1 nuclease. When MMTV-P RNA was annealed with GR DNA and assayed by digestion with pancreatic ribonuclease or S1 nuclease, the extent of hybridization was 11% greater by S1 (unpublished observation). This might indicate that ribonuclease is capable of digesting some areas of RNA hybrids. Less rigorous conditions of digestion with ribonuclease have been used by others, but with no increment in the extent of hybridization beyond 70% (Neiman, 1972; Delovitch and Baglioni, 1973; Gelderman et al., 1971).

Virus specific sequences in cell DNA may be an incomplete representation of the viral genome in certain uninfected tissues. For example, RSV and AMV RNA react only to a limited extent with normal chicken cell DNA, while they can hybridize up to 70% with DNA from infected cells or tumors (Neiman, 1972; Shoyab et al., 1973). This possibility for MMTV is discussed in Section II.

Each of the factors presented above must be taken into consideration when evaluating results obtained by RNA-DNA hybridization in DNA excess. Because of the complicated kinetics involved in such reactions, the normalization or correction of data is potentially hazardous and can

result in erroneous conclusions. Also, copy numbers estimated in relation to standard RNA molecules with entirely different nucleotide compositions are dubious. Accurate estimation of copy numbers can be made using RNA-DNA hybridization techniques, however this requires extensive analysis at varying DNA/RNA ratios (Tereba, 1973).

II. Evaluation of Experimental Results

Three factors appear to play a major role in the genesis of mammary tumors in mice: hormonal stimulation, the genetic constitution of the mouse and a mammary tumor agent(s). The responsiveness of mammary tumors to hormonal stimulation is clearly evidenced by the natural development of mammary tumors only in female mice, the increased incidence of such tumors in breeding over virgin females, and the induction of mammary tumors by the artificial administration of hormones to male or female mice from low mammary tumor incidence mouse strains. The relationship between a mouse's genetic constitution and the mammary tumor virus(es) with respect to the development of mammary tumors is unclear. Several strains of mice which were bred for their ability to develop a high incidence of mammary tumors have been found to harbor a mammary tumor virus(es) which is normally transmitted through the milk of lactating females to their offspring. In addition, these mouse strains may contain MMT virus-like information which is transferred via the gametes, since removal of the milk agent(s) can reduce but not eliminate the occurrence of mammary tumors. Other strains of mice which were bred for their low incidence of mammary tumors do not normally transmit a milk born virus, yet recent experiments have indicated the existence and possible genetic transmission of MMTV genes in these mice also.

In view of these findings, it was of interest to compare mice with a high or low incidence of mammary tumors, using nucleic acid

hybridization techniques, to see if both types of mice contain MMTV-specific sequences in their cellular DNA. If so, to examine the viral gene frequency, the extent of viral gene representation and the degree of homology of these sequences among the different mouse strains.

The DNA from all strains of mice examined to date has been found to contain MMTV-specific sequences. Using a double stranded DNA synthesized by MMTV RNA directed DNA polymerase, Varmus et al. (1972a) were able to find MMT viral sequences in two inbred strains of mice (GR, C57BL/6) which differed in their incidence of mammary tumors. These observations have been confirmed by the present study and extended to include other mouse strains. Mice with a high (GR, RIII, C3H), intermediate (C3HeB/Fe) or low (BALB/c, C57BL/6) incidence of mammary tumors were found to contain at least a portion of MMTV specific sequences, as indicated by the hybridization of MMTV double or single stranded transcripts, or MMTV RNA with DNA from each of these mouse strains (Figures 3,8,13). Virus specific sequences appeared to be in close association, probably covalently linked, with the cell DNA, since reassociation of MMTV DS-DNA was equally accelerated by total cell DNA, "networks" prepared from high molecular weight DNA and heavy DNA isolated in a neutral sucrose gradient (Tables II, III). Further evidence for MMTV specific sequences in mouse cell DNA has recently been provided by the hybridization of MMTV cDNA with DNA from NIH Swiss, C57BL X Af (Scolnick et al., 1974), ferral mice (Morris, unpublished observation; Scolnick et al., 1974) and Asian mice (M. caroli and M. cervicolor;

Ringold, unpublished observation). Additional indirect evidence has also come from the hybridization of MMTV cDNA to RNA from DBA/2, 129, I and C58 lactating mammary glands (Varmus et al., 1973b). The occurrence of MMT virus-specific sequences in the DNA of at least 16 mouse strains, irrespective of their incidence of mammary tumors, would support the predication for such sequences in all mice (Bentvelzen, 1972).

Since all mice probably contain MMTV sequences, the different incidence of malignancies among inbred mouse strains cannot be attributed to a complete presence or absence of viral information. Therefore, the possibility of a gene dosage effect was examined. Initial experiments, based upon the kinetics of reassociation of MMTV DS-DNA in the presence of mouse cell DNA, showed no significant differences in the number of viral copies for high and low incidence mouse strains (Table II). Although lower numbers of viral copies were estimated by the kinetics of hybridization with MMTV cDNA, strains RIII, C3H, C3HeB/Fe and C57BL/6 still displayed very little variation in gene frequency (13-17 copies/diploid cell, Table V). Differences in viral copy numbers were found, however, when MMTV cDNA was annealed with DNA from BALB/c or GR mouse strains. BALB/c mice were consistantly low with 9 copies/diploid cell, while GR mice had approximately 2-4 times more viral copies per cell than other mouse strains (32, Table V). Differences in copy numbers have also been resolved among mouse strains for MuLV with DNA (Chattopadhyay et al., 1974; Lowy et al., 1974), which could not

be seen by analysis with viral DS-DNA (Gelb et al., 1971a; 1973). Changes in copy numbers between uninfected and infected cells were likewise found with ASV cDNA (Schincariol and Joklik, 1973; Varmus et al., 1974) and not with DS-DNA (Varmus et al., 1973a). The contrasting results obtained using DS-DNA or cDNA as hybridization reagents are most likely attributable to the limited representation of viral genomes in virus specific DS-DNA.

The apparent disparity in gene frequency between GR and other strains of mice was further established by the differences in the rate and extent of hybridization of MMTV-S RNA with DNA from these mouse strains (Figure 12). Hybridization was greatest with GR (51%), intermediate with RIII, C3H and C3HeB/Fe (46%) and least with BALB/c (40%). Even greater differences were observed between GR and BALB/c strains of mice when MMTV-P or MMTV-O RNA was annealed with their DNA (Figure 13). Both types of RNA hybridized 20-30% more with GR than with BALB/c DNA, indicating more than a two fold difference in virus specific sequences between GR and BALB/c mice.

The high incidence of mammary tumors in GR mice and low incidence in BALB/c correspond to their high and low frequency of viral sequences. But no differences in viral copy number were found between other high (RIII, C3H) and low incidence (C3HeB/Fe, C57BL/6) mouse strains, indicating that gene frequency alone cannot account for the variation in mammary tumor incidience. Since DS-DNA and cDNA are incomplete viral transcripts, it is possible that the sequences detected by these hybridization reagents in low incidence strains of mice represent

just a portion of the viral genome, and that complete MMTV sequences are found only in high incidence mouse strains infected with milk born virus. For example, Neiman et al. (1974) have shown that normal cells contain sequences homologous to RSV but do not contain complete viral genomes. Additional viral sequences were found, however, in the DNA of RSV infected cells.

Perhaps the best method for estimating the fraction of a viral genome present in cell DNA is the hybridization of viral RNA with a large excess of cellular DNA. When RNA from MMTV-P or MMTV-O was hybridized with an excess of GR cell DNA, each type of RNA reacted with the same kinetics and to the same extent (approximately 70%, Figure 13). Because of the unfavorable kinetics involved in RNA:DNA hybridization in DNA excess, 70-80% may be the maximum extent of annealing possible with this technique, even when the entire viral genome is present in the cellular DNA. This supposition is supported by the following: (a) Endogenous leukosis viruses (subgroup E) are transmitted genetically in chicken cells and can be induced from normal chicken cells by ionizing irradiation, chemical carcinogens or mutagens (Weiss et al., 1971). Yet hybridization of subgroup E (RAV-0) virus RNA with normal chicken cell DNA is complete at 74% hybridization (Neiman et al., 1974). (b) The endogenous guinea pig oncornavirus (GPV), also genetically transmitted, can be activated from normal guinea pig cells with bromodeoxyuridine (Nayak and Murray, 1973). But RNA isolated from GPV does not hybridize beyond 70-80% with normal guinea pig DNA (Nayak, 1974). (c) Complete sarcoma viral genomes are

reportedly contained in RSV infected cells, since DNA fragments from infected cells have been used to infect normal chicken cells (Hill and Hillova, 1972). However, when RSV RNA is hybridized with RSV infected chicken cell DNA, the reaction is limited to 70-75% hybridization. If one accepts values of 70-80% hybridization as the maximum extent of hybridization possible, due to the limitations of RNA:DNA hybridization in DNA excess, then from Figure 13a it would appear that GR mice contain complete sequences for MMTV-P. The gametal transmission of MMTV-P in GR mice (Mulbach, 1965) would also favor an argument for complete MMTV genome representation in the cellular DNA of these mice. The similar extent of hybridization achieved with MMTV-O RNA and GR cell DNA is surprising in view of the possible differences between MMTV-O and MMTV-P viruses (see below) and would connote the existence of a second complete MMTV genome in GR cell DNA. Possible variation in the MMTV genetic content of GR mice has been suggested by Nandi and Helmich (1975a) to explain the large number of hyperplastic alveolar nodules appearing along with pregnancy responsive malignancies in this mouse strain. Furthermore, genetic crosses between GR and BALB/c mice have established that two independently segregating, dominant genes are concerned with MMTV transmission in GR mice, and either alone is adequate for tumor formation (Nandi and Helmich, 1974b).

A more rigorous interpretation of the hybridization studies with MMTV-P and MMTV-O RNA would be limited to the sequences actually hybridized. In which case one can only conclude that a majority of

the viral sequences are present in GR cell DNA for both MMTV-P and MMTV-O viruses.

Although MMTV-O RNA annealed to 70% with GR DNA, it hybridized to only 52% with BALB/c cell DNA (Figure 13b). In view of the different effective sequence ratios for these two strains of mice, it might be surmised that the lower extent of hybridization achieved with BALB/c DNA was an effect of inadequate sequence ratio, rather than one of incomplete viral genome representation in BALB/c cells. However, since the original source of MMTV-O virus was from a spontaneous tumor in a European sub-line of BALB/c mice, it is possible (1) that these mice harbor a different variant of MMTV than American BALB/c mice or (2) that only tumor tissue contains complete viral genomes. In either case the decreased extent of hybridization observed with American strain BALB/c DNA could result from incomplete MMTV-O virus sequence representation in these mice.

The issue of viral sequence representation in normal versus tumor tissue was examined for BALB/c mice by hybridizing MMTV-O and MMTV-P viral RNA with DNA obtained from a transplanted BALB/c mammary tumor reputed to have been originally induced with MMTV-O (Hageman, personal communication). The rate and final extent of hybridization for each type of RNA with tumor cell DNA (Figure 13c) was markedly increased over the annealing with normal BALB/c DNA (Figure 13b). The 10-20% increase in hybridization denotes a greater amount of MMTV specific sequences in tumor cells when compared to normal cell DNA. Whether this increase represents additional virus sequences or an amplification

of sequences also present in normal cells cannot be determined by these data. The possible difference between American and European substrains of BALB/c cannot be overlooked as an alternative explanation for additional hybridization occurring between MMTV RNA and European BALB/c tumor cell DNA. Until greater DNA:RNA mass ratios are attempted with American BALB/c DNA, and the DNA from normal and tumor tissue of European BALB/c sublines has been more extensively tested, these possibilities remain at issue.

The extent of MMTV-S genome representation in mouse cell DNA is also unclear. MMTV-S did not hybridize extensively to cellular DNA from mouse strains with a high or a low incidence of mammary tumors (Figure 12). The maximum extent of hybridization was 40-50%. This result can be indicative of inadequate initial sequence ratios or could denote incomplete MMTV-S genome representation in DNA of normal mouse tissues. Since MMTV-S is apparently transmitted only through the milk of lactating females to their offspring, it is feasible that this mammary tumor virus infects just target cells and therefore exists as a "provirus" only in the DNA of special tissues such as mammary glands or mammary tumors. Until MMTV-S RNA has been hybridized with mouse cell DNA at higher mass ratios and is annealed to DNA from possible target tissues, the extent of MMTV-S genome representation in mouse cell DNA remains inconclusive.

The hybridization of RIII milk virus DNA transcripts or MMTV-P, MMTV-O and MMTV-S RNA with mouse cell DNA indicated some sequence homology between these viruses and MMTV genes endogenous to the mouse genome. The degree of homology is not complete, however, since

variation among endogenous viral sequences was indicated from the thermal stability of hybrids formed between MMTV cDNA and DNA from each strain of mice (Figure 11). Because the MMT viral sequences present in the DNA from each of the mouse strains could not be compared directly, they were compared in relationship to the hybrids formed between cDNA transcribed from RIII milk virus and the DNA from RIII mice. These hybrids were the most stable, indicating extensive homology between the viral sequences endogenous to RIII mice and virus released into the milk of lactating RIII mice. The MMT viral sequences in C3H, C3HeB/Fe and BALB/c DNA appeared similar to each other, but were slightly different than those found in RIII cell DNA (3% mismatching). The MMTV sequences in C57BL/6 cell DNA varied slightly more (4.3% mismatching), while those in GR mice showed the greatest difference (5.4% mismatching). The similarity among sequences detected in C3H, C3HeB/Fe and BALB/c mice is interesting since these strains were all originally derived from Bagg albino mice. This reflects a closer relationship between viral sequences in these mice than viral sequences endogenous to other strains of mice.

Differences among MMT viruses have been noted with respect to the type of mammary tumors which develop following infection. European mouse strains (RIII, GR, DD) transmit virus which causes pregnancy responsive malignancies or plaques, while American mouse strains (C3H, DBA, A) generally carry a virus which causes hyperplastic alveolar nodules (HAN). C3H mice harbor another MMTV variant which causes HAN, but unlike the HAN induced by the milk born virus (MMTV-S), these HAN rarely develop into tumors. Differences in the antigenicity

for viruses obtained from C3H, C3HeB/Fe, BALB/c and GR mice have also been reported (Blair, 1971; Hageman et al., 1972). Therefore one would expect to find some nucleic acid sequence divergence among the viruses obtained from the various mouse strains. But the heterogenity observed among viral sequences in normal mouse cell DNA may have no relationship to the sequence homology among the MMT viruses actually responsible for tumor formation in mice. For example, the large differences in the stability of MMTV cDNA hybrids formed with GR and RIII cell DNA were not observed between hybrids formed with MMTV cDNA and RNA from virus producing GR and RIII lactating mammary glands or mammary tumors (Varmus et al., 1973b).

The most convincing evidence for sequence variation among mammary tumor viruses was obtained for MMTV-O and MMTV-P. When annealed at the same DNA:RNA mass ratio, MMTV-O RNA hybridized faster and farther than MMTV-P RNA with BALB/c DNA (Figure 13b). This indicates some sequence differences between MMTV-O and MMTV-P viruses, since under identical annealing conditions, homologous viruses should hybridize with the same kinetics. The denaturation profiles from the hybrids formed with MMTV-P or MMTV-O RNA and GR or BALB/c DNA suggest differences in the endogenous viral sequences of these mice, since hybrids formed with each type of viral RNA denatured over a broader temperature range with BALB/c than with GR cell DNA.

The question of homology among mammary tumor viral sequences in mouse cell DNA and in viruses released from the various mouse strains requires a more thorough inquiry. The competition hybridization

or oligonucleotide finger print analysis used to examine homologies among avian oncornaviruses might be a logical approach to this problem (Neiman et al., 1974; Wright and Neiman, 1974; Beeman et al., 1974).

MMTV specific sequences were found at approximately the same frequency in the DNA from mice with a high or a low incidence of mammary tumors. If these sequences are indicative of the presence of complete viral genomes, then the regulation of mammary tumor development is most likely to occur at the level of transcription or translation of viral genes. The transcriptional control of MMTV genes has been demonstrated with two clonal cell lines from an MMTV producing mammary tumor cell line (Parks and Scolnick, 1973). Each of these clones had similar growth and morphological properties, possessed the same level of virus specific information in their genomes (as determined by hybridization with MMTV cDNA), but varied in their expression of MMT viral genes. Further evidence to suggest somatic gene regulation of MMTV genes comes from the spontaneous mutation of C3H to C3H-A^{vy} (Vlahakis et al., 1973). A change in coat color was accompanied by an increased mammary tumor incidence among foster nursed mice. The incidence of mammary tumors could be reduced to 40% in C3H mice by foster nursing, but foster nursed C3H-A^{vy} mice and their offspring continued to have a 90% incidence of mammary tumors. The higher mammary tumor incidence in C3H-A^{vy}f mice could result from a mutation in genes controling the expression of endogenous viral sequences (MMTV-L). Although GR mice consistantly showed a higher frequency of MMTV gene sequences than other mouse strains, the high incidence

of mammary tumors in these mice may also be due to the effect of gene regulation at a transcriptional level. Varmus et al. (1973b) have shown that the level of transcription of MMT viral genes is much lower in spleen and liver than in mammary tissues of GR mice, indicating some somatic gene regulation of viral sequence expression. Further investigations by Varmus et al. (1973b) showed that the expression of MMTV specific sequences varies according to the type of nonvirus producing tissue and between lactating mammary glands from high and low incidence strains of mice. These results indicate that cellular differentiation together with the genetic constitution of the mouse strain determine the hormonal responsiveness and influence the transcription of MMTV genes in mouse tissues.

In summary, it appears that all mouse strains probably contain some MMTV-specific sequences covalently linked to their cell DNA, regardless of their incidence of mammary tumors. There seems to be some variation among MMTV sequences found in the DNA of the different mouse strains and between the genomes of the mammary tumor viruses released from GR and BALB/c tumor tissues. The extent of MMT viral genome representation in mouse cell DNA is uncertain, although evidence strongly favors the complete representation of MMTV-P and MMTV-O in GR cell DNA. No definite correlation can be made between viral copy number and the incidence of mammary tumors. These results support the prediction made by Bentvelzen for the genetic transmission of MMTV viral genes in all mice. Whether the MMTV specific sequences detected by nucleic acid hybridization represent a germinal provirus, an oncogene-virogene, or a protovirus has not been determined.

APPENDIX

A. Determination of Specific Activity for [^{32}P]cDNA:

Reactions for the preparation of [^{32}P]cDNA are designed according to the final specific activity desired for a particular cDNA. Calculations are based on the assumption that all four deoxynucelotide triphosphates (dNTPs) are incorporated equally into viral cDNA. The following example shows the calculations used for the preparation of MMTV [^{32}P] cDNA having a specific activity of 2.5 X 10^5 cpm/ug.

(a)	specific activity of [^{32}P]dNTP	=	[^{32}P]dATP 28.6 Ci/mM 2.2 Ci/ml
(b)	concentration of [^{32}P]dNTP	=	7.7 X 10^{-5} mM/ml
(c)	number of cpm/µl* of [^{32}P]dNTP	=	2.9 X 10^6 cpm/µl
(d)	reaction volume	=	10 ml
(e)	concentration of each dNTP in reaction	=	5 X 10^{-6}M
(f)	average number of µgm/µM for each dNTP	=	327 µg/µM
(g)	number of cpm/µg desired	=	2.5 X 10^5 cpm/µg

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* The number of cpm/µl was determined by spotting 1 µl of [^{32}P]dNTP on a filter, drying and counting in toluene-liquifluor solution in a Beckman Scintillation counter.

(h)	number of μM of each dNTP required per reaction	=	(10 ml) X (5×10^{-6} mM/ml) = 5×10^{-2} μM
(i)	total number of μM of dNTP required per reaction	=	(5×10^{-2} μM) X (4) = 200 μM
(j)	total number of μg DNA possible	=	(200 μM) X (327 μg/μM) = 6.5×10^{4} μg
(k)	total number of cpm necessary to obtain desired specific activity	=	(2.5×10^{5} cpm/μg) X (6.5×10^{4} μg) = 1.6×10^{7} cpm
(l)	number of μl of [^{32}P]dNTP to use per reaction	=	$\frac{1.6 \times 10^{7} \text{ cpm}}{2.9 \times 10^{6} \text{ cpm/μl}}$ = 5.5 μl of [^{32}P]dATP/10 ml reaction
(m)	number of mM of [^{32}P]dNTP added/reaction	=	(7.7×10^{-5} mM/ml) X (5.5×10^{-3} ml) = 4.24×10^{-7} mM

Cold dATP must be added to this reaction to equal a final concentration of 5×10^{-6} M, since the amount of [^{32}P]dATP used was not sufficient to reach this concentration.

B. Determination of the Specific Activity for MMTV [^{3}H]DS-DNA and MMTV [^{3}H]cDNA:

(a) specific activity of [^{3}H] deoxynucleotide triphosphate (dNTP) = 20 Ci/mM; 0.5 mCi/ml

(b) volume of [^{3}H]dNTP used in reaction = 0.5 ml

(c) number of mCi used = (0.5 mCi/ml) X (0.5 ml) = 0.25 mCi

(d) number of mM used = $\frac{2.5 \times 10^{-2} \text{Ci}}{20 \text{ Ci/mM}}$ = 1.25×10^{-5} mM

(e) average number of µgm/µM for each dNTP = 327 µg/µM

(f) total number of µg of DNA possible = (327 µg/µM) X (1.25×10^{-2} µM) = 4.1 µg

(g) number of cpm/µl of [^{3}H]dNTP = 2.3×10^5 cpm/µl

(h) total number of cpm used = (2.3×10^5 cpm/µl) X (5×10^2 µl) = 1.5×10^8 cpm

(i) number of cpm/µg = $\frac{1.5 \times 10^8 \text{cpm}}{4.1 \text{ µg}}$ = 2.8×10^7 cpm/µg

(j) since only 1/4 of the dNTPs in DNA could be this particular dNTP the final number of cpm/µgm is divided by 4 = 7 X 10^6 cpm/µg.

Similar calculations are made for the other [^{3}H]dNTPs used in the reaction mixture. The final specific activity for the [^{3}H]DS-DNA or [^{3}H]cDNA is taken as the sum of the individual specific activities.

(k) the specific activity
for [^{3}H]dATP = 2.97 X 10^7 cpm/µg
for [^{3}H]dTTP = 2 X 10^7 cpm/µg

(l) the total specific activity for this preparation of viral DNA = 3 x 10^7 cpm/µg

C. <u>The Number of Viral Genome Equivalents Per Diploid Mouse Cell Calculated From the Reassociation of MMTV DS-DNA in the Presence of Mouse Cell DNA</u>:

(a) molecular weight of mouse cell DNA = 4 X 10^{12} daltons/diploid cell

(b) the complexity of MMTV DS-DNA = 1.2 X 10^6 daltons

(c) ratio of DNA for one copy of viral DNA per diploid mouse cell = $\frac{4 \times 10^{12} \text{ daltons}}{1.2 \times 10^6 \text{ daltons}} = 3.33 \times 10^6$

(d) ratio of mouse/viral DNA used in reaction = $\frac{4 \times 10^{-3}\text{gm}}{1.3 \times 10^{-9}\text{gm}} = 3.07 \times 10^{6}$

(e) number of complementary $[^{3}H]$DS-DNA copies added per diploid mouse cell = $\frac{3.1 \times 10^{6}}{3.3 \times 10^{6}} = 0.94$

(f) relative rate of acceleration = $\frac{\text{Cot 1/2 for MMTV DS-DNA with salmon sperm DNA}}{\text{Cot 1/2 for MMTV DS-DNA with mouse cell DNA}}$

(g) Number of viral genome equivalents per diploid mouse cell = (relative rate of accelearation) X (number of MMTV DS-DNA copies added per mouse cell) - (number of MMTV DS-DNA copies added per mouse cell).

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